Methionine sulfoxide reductases of *Aspergillus nidulans*

Dissertation

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Zusammenfassung

Oxidation von Methionin verringert die Hydrophobizität dieser Aminosäure, was in Proteinen zu Veränderungen der Struktur führen kann. Es wird spekuliert, dass die Akkumulation solcher oxidierten Proteine ein wichtiger Mechanismus im Alterungsprozess ist, wie auch in Krankheiten wie Diabetes und in neurodegenerativen Erkrankungen. In diesem Zusammenhang haben Methioninsulfoxidreduktasen eine wichtige Schutzfunktion, indem sie Zellen vor oxidativer Schädigung bewahren können. Oxidiertes Methionin-Sulfoxid existiert in zwei Diastereomeren, dem Methionin-S-Sulfoxid und dem Methionin-R-Sulfoxid. In allen bisher untersuchten Organismen findet man eine Klasse von MSR Enzymen (MsrA), die sowohl freies Met-S-O als auch Met-S-O in Proteinen reduzieren kann. Im Gegensatz dazu bedarf es zur Reduktion von Met-R-O zweier Enzymklassen, eine zur Reparatur von freiem Met-R-O (fRMsr) und eine weitere (MsrB) für proteingebundenes Met-R-O. Nach dieser Substratspezifität lassen sich also drei Typen von Msr-Enzymen unterscheiden: MsrA, MsrB und fRMsr. Die Zahl der Isoformen innerhalb dieser drei Gruppen variiert zwischen verschiedenen Spezies, aber zumindest findet sich in jedem, bisher untersuchten Organismus mindestens ein MsrA und ein MsrB-Enzym. Der dritte Enzymtyp (fRMsr) konnte dagegen bisher nur in einzelligen Organismen nachgewiesen werden. Der Genus *Aspergillus* umfasst sowohl Pathogene für Mensch und Pflanzen, als auch Arten die in der Nahrungsproduktion und der Herstellung von industriellen Enzymen Verwendung finden. Bis zum Beginn dieser Arbeit sind keine Msr-Enzyme aus Aspergillen oder aus anderen mehrzelligen Pilzen isoliert und charakterisiert worden.

Die vorliegende Arbeit konzentriert sich auf zwei zentrale Fragen: 1. Wie können mehrzellige Organismen, für die bisher kein fRMsr-Enzym bekannt war mit freiem Met-R-O umgehen? 2. Sind MsrA und MsrB bezüglich ihrer Peptidsubstrate unselektiv oder beeinflusst der Sequenz-Kontext eines oxidierten Met-O seine Reduktion?

In dieser Arbeit gelang es drei Msr-Enzyme in *A. nidulans* zu identifizieren, AnMsrA, AnMsrB und AnfRMsr und ihre Aktivität gegenüber freiem sowie peptidgebundenem Met-O mittels RP-HPLC und Kapillarelektrophorese zu untersuchen. Rekombinant exprimiertes AnMsrA reduzierte sowohl freies Met-S-O als auch Met-S-O in Peptiden. Dagegen war AnfRMsr spezifisch für freies Met-R-O und AnMsrB für Met-R-SO in Peptiden. Alle drei Enzyme hatten maximale Aktivität bei pH 8,0 und waren nahezu inaktiv bei einem pH-Wert unter 6,0. Ortsspezifische Mutagenese wurde eingesetzt um Cystein-Reste zu identifizieren,

die an der enzymatischen Aktivität beteiligt sind. Während sowohl AnMsrA als auch AnfRMsr zwei Cysteine zur Reduktion von Met-O benötigten, war in AnMsrB nur ein Cystein nötig für die Reduktion von Met-O in Peptiden. Nach bisherigem Kenntnisstand lassen sich Organismen nach der Anzahl der MSr-Familien gruppieren: (i) Die erste Gruppe umfasst Organismen mit nur zwei Msra-Typen, MsrA und MsrB, hier finden sich der Mensch sowie alle anderen Säuger und höheren Eukaryota. (ii) Die zweite Gruppe zeichnet sich dadurch aus, dass neben MsrA und MsrB noch fRMsr zur Reduktion von Methioninsulfoxid zur Verfügung steht. In dieser Gruppe findet man Bakterien und einzellige Eukaroyta wie die Hefe. Die vorliegende Arbeit zeigt, dass auch *A.nidulans* als niederer, aber mehrzelliger Eukaryot in diese Gruppe gehört.

Knockout-Stämme von A. nidulans, bei denen einzelne Msr-Gene ausgeschaltet wurden, zeigten in Inhibitionszonen-Assays eine erhöhte Sensitivität gegenüber oxidativem Stress durch drei getestete Verbindungen, H_2O_2, Chloramin-T and Menadion. Eine mögliche Bedeutung der drei Msr-Gene in *A.nidulans* wird auch dadurch impliziert, dass alle drei Msr-Gene unter oxidativen Stressbedingungen stärker transkribiert werden als unter Kontrollbedingungen.

Um den Einfluss der Peptidsequenz auf die Aktivität von MsrA und MsrB-Enzyme zu überprüfen, wurden sowohl die beiden humanen Enzyme, als auch MsrA und MsrB aus *A.nidulans* in Aktivitätsassays mit einer Serie verschiedener Peptidsubstrate untersucht. Die Variation der flankierenden Aminosäuren vor, bzw. hinter dem oxidierten Methionin ergab, dass MsrA-Enzyme sehr sensitiv gegenüber negativen Ladungen in diesen Positionen sind, während die MsrB-Enzyme weniger vom Kontext des Met-O Substrates beeinflusst wurden. Insgesamt ergaben diese Studien, dass die Reduktion von Met-O in Peptiden oder Proteinen von der benachbarten Sequenz beeinflusst wird und dass dieser Einfluss enzymabhängig ist.

Um den möglichen Mechanismus der unterschiedlichen Peptid-Selektivität in MsrA und MsrB näher zu untersuchen, wurden die aktiven Zentren beider Enzyme verglichen. Die katalytischen Zentren der beiden Enzymklassen sind etwa spiegelsymmetrisch angeordnet, wobei auffällig ist, dass negative Ladungen in MsrA Enzymen (Asp134 und Glu99 in AnMsrA) vorkommen, die in MsrB Enzymen nicht vorhanden sind. Gezielte Mutagenese dieser Aminosäuren in MsrA unterstütze die Hypothese, dass diese negativen Reste für die größere Peptidselektivität bei MsrA verantwortlich sind.

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To Fulfill the
Requirements for the Degree of
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Submitted to the Council
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by **Rabab GH Abdelaleem, M.Sc.**

born on 01.04.1982 in Giza, Egypt

Summary

Methionine oxidation changes the hydrophobic properties of Met to more hydrophilic properties, often resulting in structural alterations in proteins. It has been hypothesized that accumulation of oxidized proteins is an important mechanism of the aging process, diabetics and neurodegenerative diseases. Methionine sulfoxide reductases constitute an important defense system to protect cells against oxidative stress. Oxidized methionine occurs in the form of two diastereomers, methionine-S-sulfoxide and methionine-R-sulfoxide. In all studied organisms the S-epimer requiring one types of MSR enzyme (MsrA) which is responsible for the reduction of free Met-S-O and protein-bound Met-S-O. In contrast to the S-epimer, the reduction of the R-epimer requires two enzymes; one to repair the free Met-R-O (fRMsr) and another one for the protein bound Met-R-O (MsrB). Based on the substrate specificity, Msr enzymes exist in three types: MsrA, MsrB and fRMsr. The number of isoforms of each Msr type is widely different among different organisms, however, there are at least one MsrA and MsrB in each organism. The third enzyme (fRMsr) has been reported to be limited to unicellular organisms. The genus *Aspergillus* includes human and plant pathogens as well as beneficial species used to produce foodstuffs and industrial enzymes. Thus far, no Msrs have been characterized from any *Aspergillus* species or from other multicellular fungi.

The present thesis aimed to address two questions. First, how multicellular organisms eliminate free Met-R-O? The second question, are MsrA and MsrB have the ability to reduce MetO in any context? To address the first question, we choose *A. nidulans*, which is a key fungal model system for genetics and cell biology, as a model for the multicellular organisms. This organism provides many advantages over many organisms such as, in contrast to mammals and plants *A. nidulans* only have one isoform of each Msr type, also it is a multicellular organism which offers a more complicated system than bacteria and yeast. In order to investigate the second question, we tested the ability of both fungal and human MsrA and MsrB to reduce MetO localized in different context.

Three Msr enzymes have been successfully identified from *A. nidulans*, AnMsrA, AnMsrB and AnfRMsr. Their activity has been tested toward free MetO and peptide bound-MetO using RP-HPLC and capillary electrophoresis assays. AnMsrA was found to reduce of both free and peptide-pound Met-S-O, while fRMsr was found to be specific for free Met-R-O and MsrB for peptide-bound Met-R-O. The three enzymes showed the highest activity at pH 8.0 while at pH 6.0 or below they were almost inactive. Site-directed mutagenesis was used to

identify the catalytic Cys for each enzyme. The data showed that both of AnMsrA and AnfRMsr use two Cys to reduce MetO, while AnMsrB used only one Cys for catalysis of peptide-bound Met-R-O. According to the literature and our study on fungal Msrs, methionine sulfoxide reductase family exists in two groups based on the type of MSR. (i) The first group contains the organisms that have two types of Msr (MsrA and MsrB) which can be found in mammals and higher eukaryotes such as human. (ii) The second group contains the organisms that use three types of Msr (MsrA, MsrB, and fRMsr) to repair methionine sulfoxide and can be found not only in unicellular organisms such as bacteria and yeast also found in low eukaryotes organisms such as Aspergillus. *msr*-single knockout strains showed high sensitivity in inhibition zone assays using three oxidants (H_2O_2, Ch-T and Menadione). In addition, the observed upregulation of the three *msr* genes suggested that all Msr enzymes are involved in stress response in *A. nidulans*.

The activity of MsrA and MsrB from human and *A. nidulans* was examined toward a set of synthetic peptides in order to study the impact of MetO-neighboring amino acids on Msr activity. MsrAs were strongly impaired by acidic residues flanking MetO, whereas MsrBs were less sensitive to the flanking amino acids. The data revealed that reduction of MetO is indeed influenced by surrounding residues of Methionine.

To address the mechanism underlying the difference in peptide selectivity of AnMsrA and AnMsrB, we compared their active sites. Although, crystal structures of MsrB enzymes show no resemblance to MsrA structures, the active sites in both show approximate symmetry. Despite the mirror-like relationship between MsrA and MsrB, it is clear from the structural features that MsrBs lack the acidic residues (Asp134 and Glu99 in AnMsrA) that are present in the active site of MsrA enzymes. Site-directed mutagenesis of the negatively charged residues in MsrA supported the hypothesis, that these residues in the active center are involved in stronger peptide selectivity of MsrA.

Dedicated to my parents

Contents

1 Introduction

1.1 Oxidative stress

Cellular components in aerobic organisms are often challenged with reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). Molecular oxygen is crucial for survival of aerobic organisms, which use it for respiration and numerous other processes, but the use of oxygen is also associated with the generation of ROS (Imlay, 2008). Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species, and their elimination by protective mechanisms, referred to as antioxidants (Duracková, 2010). This imbalance leads to damage of important biomolecules such as proteins, lipids, and nucleic acids, with potential impact on the cells and the whole organism and thus contribute to the incidence or progression of numerous disorders, including atherosclerosis, neurodegeneration, cancer, diabetes and chronic inflammation (Duracková, 2010; Roberts et al., 2009).

ROS are partially reduced oxygen derivatives that are continuously generated during respiration and stand apart from other oxygen-containing molecules because of their relatively short half-life (Hernández-García et al., 2010; Avery, 2011). Free radicals participate in a large number of subsequent reactions (Fig. 1), in which other very reactive metabolites are formed. They are derived from basic radical molecules, such as superoxide anion radical, shortly superoxide $O2^{\cdot-}$, or nitric oxide (nitroxide) $NO^{\bullet}$ (here after NO). Newly formed metabolites have a great oxidative ability and they are often more reactive than their maternal molecules. Such metabolites include e.g. the most reactive hydroxyl radical, $HO^{\cdot}$, or non-radical molecules such as hydrogen peroxide, singlet oxygen, peroxynitrite or hypochlorous acid (Ďuračková 2008). While several well-described cellular defenses against oxidative stress have evolved in aerobic organisms, ROS-mediated damage can become toxic when ROS production exceeds the capacities of these defense mechanisms (Imlay, 2008; Limón-Pacheco and Gonsebatt, 2009).

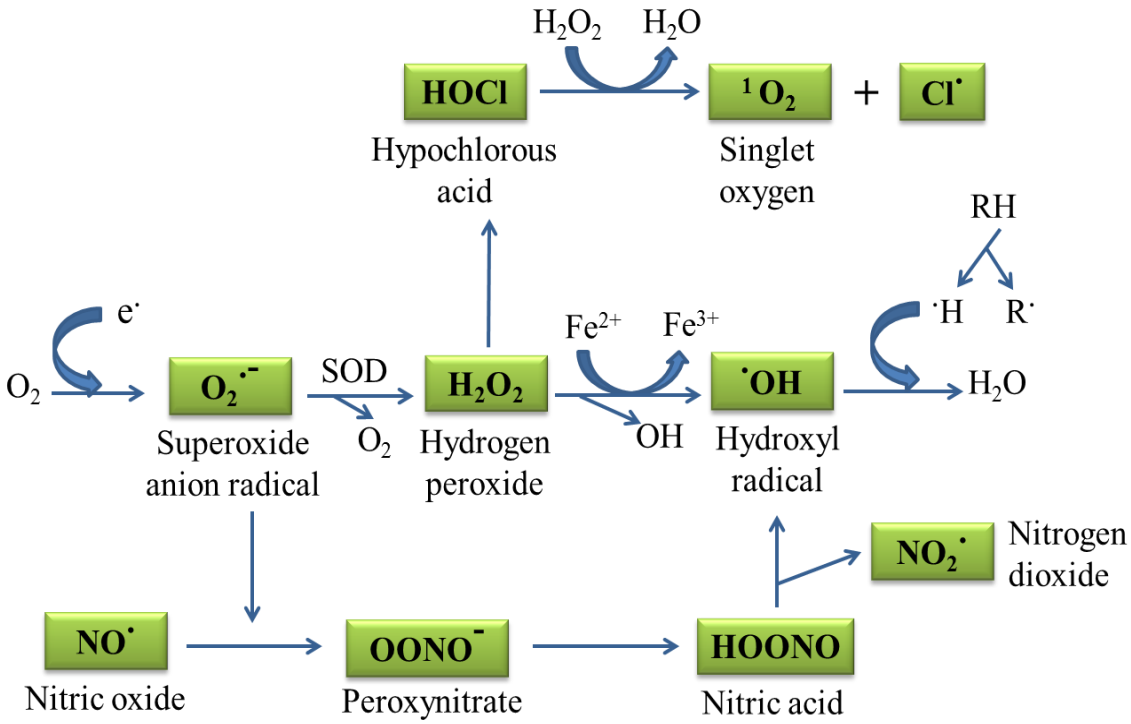

Fig. 1: Subsequent reactions of the free radicals (adapted from Ďuračková 2008).

Responding to ROS damage, organisms have evolved multiple defense systems, including low molecular weight compounds and antioxidant enzymes that protect against oxidative stress. The antioxidant system includes enzymes such as glutathione peroxidase (GPx) (Spector et al., 2001), superoxide dismutase (SOD) (Warner, 1994), catalase (Orr and Sohal, 1992), thioredoxin/thioredoxin reductase system (Trx/TrxR) (Arnér and Holmgren, 2006), methionine sulfoxide reductase (Msr) (Stadtman et al., 2002; Yermolaieva et al., 2004; Hansel et al., 2005) and many other antioxidant enzymes. Several other proteins include but are not limited to small heat shock proteins, particularly α-crystallins (Yaung et al., 2007). Amino acids in either free form or in the backbone of the peptide linkage are also exposed to oxidative modifications via ROS or nitrogen species. Together with cysteine and tryptophan, methionine is the most highly susceptible amino acid to oxidation (Vogt, 1995; Levine et al., 2000; Stadtman, 2006). Methionine oxidation changes the hydrophobic properties of Met to more hydrophilic properties, often resulting in structural alterations (Chao et al., 1997; Stadtman et al., 2003). The accumulation of oxidized proteins has been hypothesized to be an important mechanism of the aging process, diabetes and neurodegenerative diseases (Hoshi and Heinemann, 2001; Levine and Stadtman, 2001; Hou et al., 2002; Moskovitz, 2005; Petropoulos and Friguet, 2005; Cabreiro et al., 2006; Friguet, 2006; Styskal et al., 2012; Styskal et al., 2013).

1.2 Oxidation of methionine

Methionine and cycteine are the only two amino acids occurred naturally that contain sulfur and they are the major targets of ROS in proteins (Levine et al., 2000). Methionine is an unbranched, non-polar amino acid that contains a thioether side chain, and cysteine is a polar amino acid that contains a thiol side chain. The susceptibility of these amino acids to oxidation is a function of both their biochemical properties and their locations within a particular protein's tertiary structure. Sulfur is just below oxygen in the periodic table. Thus, it is a larger, less electronegative element than oxygen. Sulfur's weak nuclear attraction for its outermost electrons makes the thioether group of methionine nucleophilic. Unlike the reactive functional groups of other nucleophiles, methionine`s sulfur atom cannot be protonated. Hence, it is the most potent nucleophile in proteins at acidic pH (Vogt, 1995).

The first stage of methionine oxidation leads to sulfoxide (MetO) a biologically occurring product containing an oxygen atom double-bonded to the sulfur atom (Fig. 2). The next step, formation of the methionine sulfone, requires more drastic chemical attack. Methionine sulfone contains two oxygens bonded to the sulfur atom and has rarely been found in biological systems. Met sulfones are formed in more extreme oxidant conditions following the formation of MetO. They are considered irreversible, and cannot be reduced under physiological conditions (Vogt, 1995). In contrast, MetO is readily formed relative to other post-translational modifications and can be reduced by a specific group of enzymes entitled methionine sulfoxide reductases (MSR).

Oxidation of the Met sulfur atom can form either one of two possible enantiomers; denoted as S and R (Fig. 2). With some specific exceptions, there is no general evidence to date predicting which enantiomer of MetO will be formed when the side chain of Met is oxidized. The ratio of these two enantiomers in proteins can vary considerably depending on the location of the methionine residue that is oxidized and the identity of the chemical oxidant (Sharov and Schoneich, 2000).

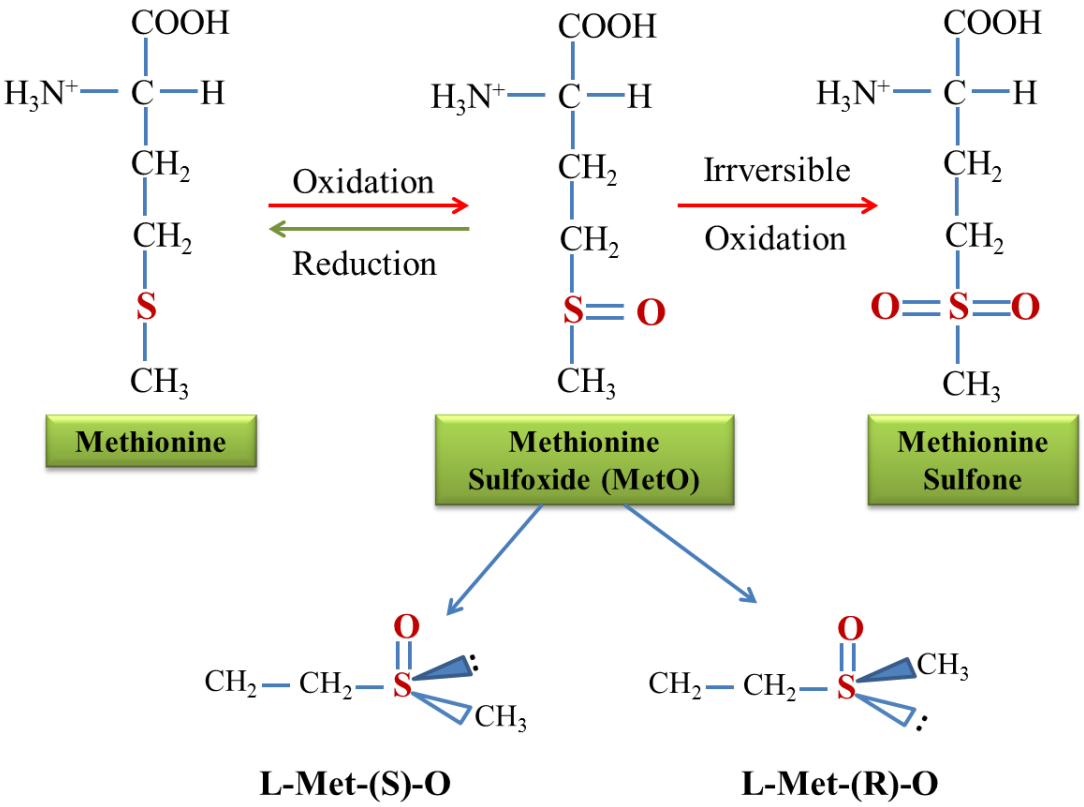

Fig. 2: Methionine oxidation products and the enantiomers of methionine sulfoxide

1.3 Methionine sulfoxide reductase family

In any living cell, methionine exist in two forms; as a free amino acid and as a residue in protein and both forms are sensitive to oxidation. Oxidized methionine occurs in the form of two diastereomers, methionine-S-sulfoxide and methionine-R-sulfoxide (Stadtman, 2006). In all studied organisms the S-epimer requiring one types of MSR enzyme (MsrA) which is responsible for the reduction of free Met-S-O and protein-bound Met-S-O. In contrast to the S-epimer, the reduction of the R-epimer requires two enzymes; one to repair the free Met-R-O (fRMsr) and another one for the protein bound Met-R-O (MsrB). Methionine sulfoxide reductases (Msr) are thiol oxidoreductases that exist ubiquitously in most living organisms (Stadtman, 2004; Weissbach et al., 2005).

Methionine sulfoxide reductases constitute an important defense system to protect cells against oxidative stress (Hansel et al., 2005; Kim and Gladyshev, 2007). Msr enzymes are widely distributed in all organisms from bacteria to humans (Delaye et al., 2007; Kryukov et al., 2002).The *msr* genes are ubiquitously expressed in many organisms, and highly expressed

in the liver and kidneys of mammals (Moskovitz et al., 1996). They have been distributed widely in tissues and are present in multiple sites of the eye. Msrs might be cooperating with other proteins to protect the cells from oxidative-stress-induced cell injury (Yermolaieva et al., 2004; Sreekumar et al., 2005). All Msrs characterized so far share the same catalytic mechanism, based on the genomic, biochemical, kinetic and structural analyses involving sulfenic acid chemistry (Boschi-Muller et al., 2000; Gruez et al., 2010). The first Msr enzyme was discovered 34 years ago is MsrA. However, the first report concerning MsrB published in 2001 identified from *E. coli* (Grimaud et al., 2001), followed by mouse (Moskovitz et al., 2002) and human (Jung et al., 2002). Recently, Msrs were investigated and characterized from a variety of organisms, new forms were discovered. Presently MSR enzymes can be divided into three large groups according to their substrate specificity and family clustering.

1.3.1 Methionine sulfoxide reductase A

It was found that MsrA enzyme can restore the function of oxidized ribosomal protein L12 in *E. coli* (Brot et al., 1981). This enzyme was also found to restore the activity of oxidized α-1-proteinase inhibitor by reducing methionine sulfoxides in this protein (Abrams et al., 1981). The MsrA gene was cloned in early 1990s from *Escherichia coli* (Rahman et al., 1992) and later the bovine MsrA gene was also cloned (Moskovitz et al., 1996); the corresponding protein was found to stereoselectively reduce the S form of free or protein-bounded methionine sulfoxide. This protein is the only known enzyme capable of reducing Met-S-O (Lee et al., 2009; Boschi-Muller et al., 2008; Sharov et al., 1999), but it can also reduce other compounds such as N-acetyl-methionine-S-sulfoxide, dimethyl sulfoxide, ethionine-S-sulfoxide, S-sulindac, S-sulforaphane, etc. (Moskovitz et al., 1996; Etienne et al., 2003; Weissbach et al., 2005). Some reports showed MsrA in a selenoprotein form in some lower organisms, such as green algae and some bacteria, wherein it utilizes catalytic selenocysteine (Sec) in place of Cys (Kim et al., 2008).

Based on the number and positions of the cysteine residues proposed to be involved in the catalytic mechanism, MsrAs can be clustered in 3 subsets (Kaufmann et al., 2005). Group I contains enzymes that use three cysteine residues in their catalytic mechanism. These enzymes systematically have a long N-terminal extension with respect to the MsrAs from other groups. The *E. coli*, bovine and human MsrAs are three examples of group I. Group II contains enzymes that use two cysteines, the *Mycobacterium tuberculosis* and *Neisseria meningitides* MsrA are the examples of group II. Group III involve enzymes that also use two

cysteines but their positions are different from those belongs to group II (Boschi-Muller et al., 2001). The *Bacillus subtilis* MsrA belongs to this class.

1.3.2 Methionine sulfoxide reductase B

The first report concerning MsrB was only published in 2001 (Grimaud et al., 2001). MsrB (described previously as SelR or SelX), is specific for the reduction of protein-based Met-R-SO, but also, with low efficiency, of free Met-R-SO. In mammals, there are three MsrB proteins, including MsrB1, MsrB2, and MsrB3. All three contain Zn, coordinated by two CxxC motifs (xx could be any two residues), which stabilizes MsrB structure. Of these three enzymes, MsrB1 is a selenoprotein that contains Sec in the place of the catalytic Cys residue normally present in other MsrBs (Kryukov et al., 2002). The other two mammalian MsrBs contain cysteine in place of selenocysteine. MsrB2 (also known as CBS-1) resided in mitochondria. It has a strong affinity for Met-R-O, but could be inhibited by higher concentrations of the substrate. Among mammalian MsrBs, the human MsrB3 is unusual in that it occurred in two protein forms; MsrB3A and MsrB3B. Genomic analyses revealed that these forms were generated by alternative splicing and differed in their N-terminal sequences (Kim and Gladyshev, 2004).

MsrB enzymes separate in two groups as MsrB I and MsrB II, distinguishable from each other by the presence of two additional CxxC motifs in the MsrBI group (Kumar et al., 2002; Kaufmann et al., 2005). These four conserved residues, at positions 45, 48, 94 and 97 (*E. coli* MsrB numbering), were shown to bind a zinc atom required for the enzyme activity in SelR (Kumar et al., 2002; Kaufmann et al., 2005). *E. coli* MsrB and the human proteins are members of the MsrB I group, while *Neisseria meningitides* MsrB belongs to MsrB II (Olry et al., 2002; Lowther et al., 2002; Kaufmann et al., 2005).

1.3.3 Free methionine R-sulfoxide reductase

The third type of MSR enzymes, fRMsr, was recently discovered and it catalyzes the reduction of free Met-R-SO (Lin et al., 2007). The fRMsrs exhibit a GAF-type fold and is highly specific for its substrate, i.e., it does not act on protein-based sulfoxides. Comparative genomic analysis of fRMsr showed that its occurrence is limited to unicellular organisms, whereas multicellular organisms lack this protein. The fRMsr is the first case of a GAF domain that bears a catalytic activity (Lin et al., 2007; Le et al., 2009).

GAF domains are one of the biggest and most widespread domains found in all kingdoms of life (Anantharaman et al., 2001). The GAF term is abbreviated from the names of the first three classes of proteins recognized to contain this domain: mammalian cGMP-binding PDEs, *Anabaena* adenylyl cyclases, and *E. coli* FhlA (Aravind and Ponting, 1997). GAF domain-containing proteins are dimeric and generally arranged in tandem in modular proteins to provide a large variety of regulation functions (Zoraghi et al., 2004). GAF motifs are composed of ~110 amino acids and are present in one to four (and even partial) copies in all living organisms from archaea to mammals; they are particularly abundant in plants and bacteria (reviewed in Zoraghi et al., 2004). Though rare in human proteins (among which they are found only in phosphodiesterases (PDEs)), there are about 2,000 GAF domain-containing proteins in the nonredundant database that are predicted to contain a GAF domain (Schultz et al., 1998; Letunic et al., 2002) in which GAF domains have been shown to provide a variety of functions including binding of small molecules, protein-protein interactions (incl. dimerization), and other processes (Heikaus et al., 2009).

GAFs have been shown to be linked with gene regulation in bacteria (Aravind and Ponting, 1997), light-detection and signaling pathways in plant and cyanobacterial phytochromes (Sharrock and Quail, 1989; Montgomery and Lagarias, 2002), ethylene detection and signaling in plants (Sato-Nara et al., 1999), nitrogen fixation in bacteria (Joerger et al., 1989), the two-component sensor histidine kinase in viruses, bacteria, and plants (Kaneko et al., 2001; Urao et al., 2001), and feedback control of a cyanobacterial adenylyl cyclase by cAMP-binding (Kanacher et al., 2002). However, the vast majority of GAF domains have not been studied in any detail so that their functions and ligand-binding potentials are in general poorly understood (Heikaus et al., 2009).

1.4 Cellular localization of MSRs

The copy number of *msrA* and *msrB* orthologs varies widely among different organisms. For example, *E. coli* contains one copy each of *msrA* and *msrB*; *S. aureus,* 3 *msrA* and 1 *msrB*; *Vibrio cholerae,* 2 *msrA* and 3 *msrB*; all present in the chromosome. *Rhizobium meliloti* possesses 3 *msrA* and 3 *msrB* genes and one of each is located on a plasmid (Ezraty et al., 2005). While in mammals MsrA is encoded by a single gene and MsrB is encoded by 3 genes (Hansel et al., 2005), *Arabidopsis thaliana* contains five genes encoding for MsrA and nine genes for MsrB (Rouhier et al., 2006).

1.4.1 Methionine sulfoxide reductase A

The human MsrA gene is located on chromosome 8 and is coded by single open reading frame regulated by two distinct promoters resulting in different isoforms: the long form and short form both contain a cysteine-containing sequence and thioredoxin domain (Pascual et al., 2009; Balog et al., 2003; Hansel et al., 2005). The long form of MsrA encodes a peptide containing an N-terminal mitochondrial targeting sequence and it is transported to mitochondria (Hansel et al., 2002). The mitochondrial targeting sequence consists of ~20 amino acids and contains, as is typical for mitochondrial signal peptides (Hermann and Neupert, 2000). The short form of MsrA lacks the mitochondrial sequence, and is localized to the nucleus and cytosol (Hansel et al., 2002; Vougier et al., 2003). Except for leukemic/lymphomic cells hmsrA mRNA could be detected in all human tissues with highly varying expression levels. Maximum expression was found in human kidney and cerebellum, followed by liver (Kuschel et al., 1999).

1.4.2 Methionine sulfoxide reductase B

Contrary to a single MsrA gene, three *msrB* genes have been identified in mammals (Fig. 3) (Hansel et al., 2005; Kim and Gladyshev, 2005). The first known mammalian MsrB was also the first selenoprotein identified computationally (Lescure et al., 1999), and it is currently known as MsrB1 (Kim and Gladyshev, 2005). The second mammalian MsrB was first described as CBS-1, a human protein with high similarity to bacterial PilB. CBS-1 was also found to have MsrB activity and was later designated as MsrB2. This enzyme contains a cysteine residue in place of the selenocysteine residue found in MsrB1 and an N-terminal signal peptide that targets the protein to mitochondria (Jung et al., 2002). The third mammalian MsrB, MsrB3, also contains a cysteine residue in the active site. Human MsrB3 has two forms, MsrB3A and MsrB3B, by alternative first exon splicing. While MsrB3A contains an ER (endoplasmic reticulum) signal peptide at the N-terminus and an ER retention signal at the C-terminus, and is targeted to the ER, MsrB3B contains a signal peptide at the N-terminus and is targeted to mitochondria. By contrast, studies on mouse MsrB3 found no evidence for alternative splicing in this species (Kim and Gladyshev, 2004). Mouse MsrB3 has consecutive ER and mitochondrial targeting signals at the N-terminus. This protein is targeted to the ER, and the function of the mitochondrial signal appears to be masked by the ER signal peptide (Hansel et al., 2005).

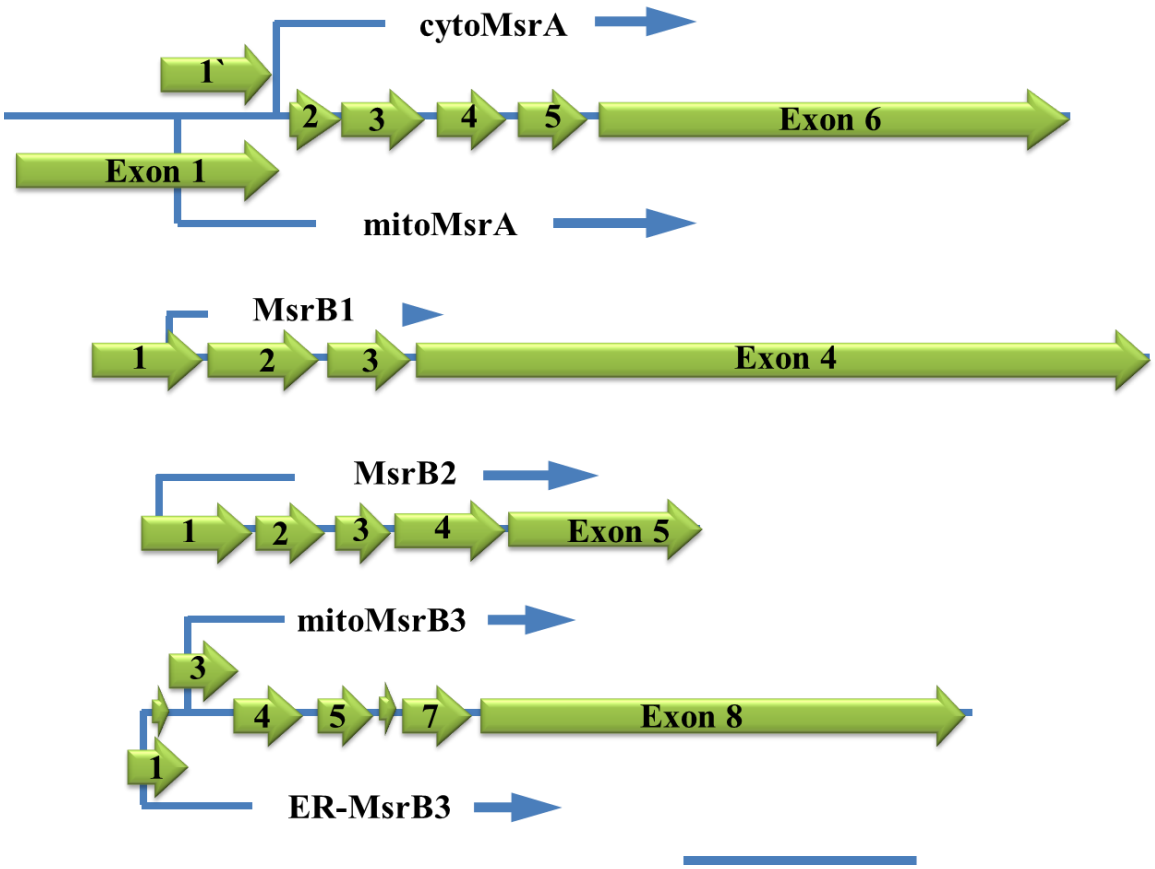

Fig. 3: Exon organization of the human MSR genes. The blue line to the bottom right represents 300 bases or 100 amino acids for both, the exons and the coding regions. Coding regions for the corresponding proteins are depicted by the black arrows above or below the exons, and the starts of these arrows are placed at the translation starts (adapted from Hansel et al., 2005).

1.5 Structure and catalytic mechanism of MSRs

All Msrs characterized so far share the same catalytic mechanism based on the genomic, biochemical, kinetic and structural analyses involving sulfenic acid chemistry (Boschi-Muller et al., 2000; Gruez et al., 2010). The catalytic mechanism of MSR includes three steps with: (1) formation of a sulfenic acid intermediate on the "catalytic" Cys residue, with concomitant release of 1 mol of Met per mol of enzyme, (2) formation of an intramonomeric disulfide bond between the catalytic Cys and the "recycling" Cys, with concomitant release of 1 mol of H_2O and; (3) reduction of the Msr disulfide bond by Trx, leading to regeneration of the reduced form of Msr (Msr_{red}) and to formation of oxidized Trx (Trx_{ox}) in a disulfide state (Fig. 4).

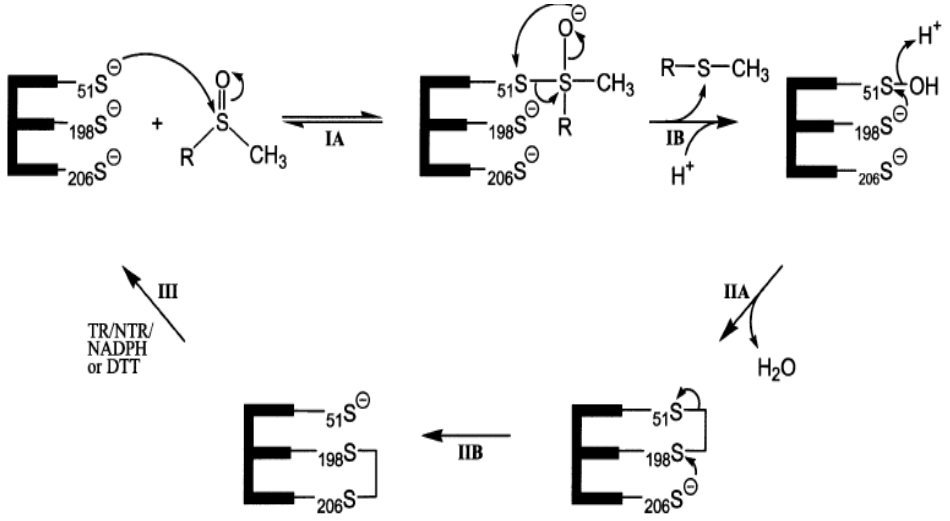

Fig. 4: Proposed reaction mechanism for *E. coli* MsrA catalysis. Attack of Cys51 on the sulfur atom of the substrate sulfoxide leads to the formation of a tetrahedral intermediate (step IA), rearrangement of which leads to the formation of a sulfenate ion, the release of a molecule of Met and the protonation of the sulfenate ion (step IB). Attack of Cys198 on the sulfur atom of the sulfenic acid intermediate leads to the formation of a transient disulfide bond between Cys51 and Cys98 and the release of a molecule of water, facilitated by acid catalysis (step IIA). Return of the active site to a fully reduced state proceeds by two thiol-disulfide exchanges via Cys206 (step IIB) and either DTT or a thioredoxin regenerating system (steps III). RSOCH3 and RSCH3 represent MetSO and Met, respectively. TR, thioredoxin reductase; NTR, NADPH thioredoxin reductase (adapted from Boschi-Muller et al., 2000).

1.5.1 Methionine sulfoxide reductase A

The structure of the MsrA proteins from *E. coli* (Tête-Favier et al., 2000), *M. tuberculosis* (Taylor et al., 2003) and bovine MsrA (Lowther et al., 2000) has been determined. The MsrA models define a single-domain protein composed of a central core around which long N- and C-terminal coils wind. The central core is composed of about 140 amino acids that describe a rolled mixed β-sheet flanked on the exterior side by three helices, with the insertions of a short α-helix and two antiparallel β-strands (Tête-Favier et al., 2000). This overall architecture is unique, since no similarly folded proteins are known (Kaufmann et al., 2005). With no apparent structural and enzymatic roles, the N-terminal end ought to be susceptible to adopt various conformations in the different MsrAs. The conformation of the C-terminal ends largely varies in the different structures, and it contains the CysB and for some of MsrA enzymes CysC (Kaufmann et al., 2005). The active site is organized around the catalytic cysteine CysA (Cys51 in cMsrA), situated at the entrance of the first α-helix α1 of the central

core. The surrounding amino acids, all strictly conserved in the MsrA sequence alignment, compose a large open basin which confers a high accessibility to CysA, adapted to the binding of bulky protein substrates.

1.5.2 Methionine sulfoxide reductase B

The *N. gonorrhoeae* MsrB (nMsrB) is mainly composed of antiparallel β-strands organized in two sheets that face each other to form a barrel-like core (Lowther et al., 2002). The loops connecting the strands are very short, and often fold in β-turns. Roughly 40 residues are found upstream from this core domain. They describe an elongated coil interrupted by two short helices, before entering the first β-strand of the core. The C-terminus is shorter and folds in two small antiparallel helices (Kaufmann et al., 2005). Contrary to MsrAs, which possess an unshared fold, the MsrB domain was found to resemble two other proteins, despite insignificant sequence identities (Lowther et al., 2002). The so-called Mss4 (PDB entry 1HRX) and TCTP (PDB entry 1H6Q) proteins, indexed as "guanine nucleotide-free chaperones" are essential for vesicular transport and involved in various human tumors (Kaufmann et al., 2005). The catalytic cysteine CysA (Cys 495 in nMsrB) is located within the strand β8, i.e., in a very constrained portion of the polypeptide chain. The side chain displays two alternative conformations and points toward the solvent from the bottom of a surface-exposed pocket that constitutes the active site (Lowther et al., 2002).

1.5.3 Free methionine R-sulfoxide reductase

The crystal structures of fRMsr from *E. coli*, *S. cerevisiae*, and *N. meningitidis* have been solved (Badger et al., 2005; Ho et al., 2000; Gruez et al., 2010). The fRMsr structure shares the overall topology of GAF domains. The three structures are described as being composed of six β-strands, four α-helices, and two prominent loops, loop 1 and loop 2, located on the surface of the protein between β2 and β3 and between β4 and β5, respectively. The fRMsr represents a unique case in which a GAF fold behaves as an enzyme and moreover as an independent folded unit not included in a modular larger protein. Like all GAF domains, fRMsr is dimeric. The two active sites within the dimer are separated from each other by 23.4 Å. Both bind an L-Met-*R*-O substrate and water molecules (Gruez et al., 2010).

1.6 The biological significance of MSRs

Oxidation of Met residues can be readily reduced by the Msr system, prevents changes resulting from Met oxidation, for example alterations protein structure, biological function, or

a combination of both possibilities. Met is a hydrophobic amino acid, and the hydrophobicity decreases when the sulfur atom is oxidized. However, the sulfoxide formation can alter the native folding and create a more hydrophobic protein (Chao et al., 1997). Msrs exert various biological functions *in vivo*. They can repair oxidized proteins, and thus, may regulate their function. The key function of Msr is to repair oxidatively damaged proteins, in which ROS oxidized methionine residues. This oxidation may interfere with protein function, because it leads to an increased negative charge and size, and consequently may result in structural changes and loss of function (Stadtman et al., 2005).

The Msr enzymes are also important for survival under oxidative stress conditions as manifested by *msrA* knockouts in various organisms (Jung et al., 2002; Moskovitz, 2005). Several reports showed that MsrA knockout mice exhibit a tip-toe walking behavior consistent with cerebellar dysfunction (Moskovitz et al., 2001), enhanced neurodegeneration in hippocampus (Pal et al., 2007), and impaired dopamine regulation (Oien et al., 2008). Similarly, MsrA deficiency causes cellular dysfunction and mitochondrial damage in cardiac myocytes under physical and oxidative stresses (Nan et al., 2010).

Along with deficiency effects, recently several studies confirmed that MsrA and MsrB are essential for the virulence of pathogenic bacteria such as *M. genitalium* (Das et al., 2012), *S. Typhimurium* (Denkel et al., 2011) and *S. gordonii* (Lei et al., 2011). That because the absence of this protein affects a range of properties like adherence (Dhandayuthapani et al., 2001; Wizemann et al., 1996; Giomarelli et al., 2006; Herzberg et al., 2005), motility (Hassouni et al., 1999) biofilm formation (Beloin et al., 2004; Kuboniwa et al., 2006), intracellular survival (Douglas et al., 2004) and in vivo survival (Dhandayuthapani et al., 2001; Hassouni et al., 1999; Mei et al., 1997). Furthermore, lack of MsrA increased protein carbonyl accumulation (Moskovitz et al., 2001). There is also evidence that Msr levels decrease in various rat tissues as they age (Petropoulos et al., 2001), supporting the link between enhanced free radical damaging action and the aging process (Stadtman et al., 2002). *E. coli* Ffh (Ezraty et al., 2004), human potassium channel hERG1 (Su et al., 2007), ShC/B channels (Ciorba et al., 1997), and activate the plasma membrane Ca-ATPase and calmodulin (Sun et al., 1999; Carruthers and Stemmer, 2008) are known to be regulated by oxidation of methionine and repair activity of Msrs. Msrs have function in maintaining redox homeostasis, repairing damaged proteins, and increasing oxidative stress resistance (Weissbach et al., 2005; Moskovitz, 2005). Additionally, MsrA can be used as a marker for isolating subpopulations of stem and progenitor cells used in regenerative medicine (Hu and El Haj, 2013).

1.7 *Aspergillus nidulans* as a model organism

Aspergillus is a genus of filamentous fungi that has more than 200 species. *Aspergillus* has diverse ecological roles. Its habitat includes soil, dust, and living or dead plant materials (Latge and Steinbach, 2009). The genus *Aspergillus* includes human and plant pathogens as well as beneficial species used to produce foodstuffs and industrial enzymes. For example, *A. fumigatus* is a deadly pathogen of immunocompromised patients; *A. flavus* is an agriculturally important toxin producer; *A. niger* and *A. oryzae* are used in industrial processes (Kapoor et al., 1999).

The *Aspergillus nidulans* belongs to the class Ascomycotina (the ascomycetes), the largest class of fungi. The ascomycetes can produce both asexual spores (conidia) and sexual spores (ascospores), the latter being produced within an ascus. The class includes fungi with wide variation of habitats. Many of them are common animal and plant pathogens; others are saprophytic and involved in the decomposition of plants and animals (Martinelli, 1994). *A. nidulans* is a saprophytic fungus. It can be isolated from soil and may also occur as mold on food. It is also able to utilize a wide range of different carbon and nitrogen sources (Ward, 1991). In the vegetative (asexual) life cycle of *A. nidulans* (Fig. 5), the germinating conidia form a network of multinucleate hyphae. Afterwards, the conidiophore elongates from the footcell, a specialized thick-walled cell within the hyphae that anchors the stalk to the growth substratum. At the tip of the conidiophore, a swollen structure is formed, which is called conidiophore vesicle. The footcell, the conidiophore and the vesicle are unseptated from each other and form a single unit. The vesicle produces a primary layer of uninucleate sterigmata (buds) called metulae. The metulae in turn bud twice to produce a second layer of uninucleate sterigmata, termed phialides. The phialides produce chains of uninucleate spores called conidia (Adams et al., 1998).

Recently, the genome sequence of *A. nidulans* was released by the Broad Institute (http://www.broad.mit.edu/annotation/fungi/aspergillus/index.html). The size of the *A. nidulans* genome is approximately 30,000 kbp. It has 8 chromosomes containing estimated 10,000-11,000 genes. *Aspergillus nidulans* is used as an experimental model species. *Aspergillus nidulans* hyphae are ~2-3 μm in diameter, and for wild-type cells the basal cell length is ~40 μm. Apical cells are usually much longer than 40 μm and have many evenly spaced nuclei. *Aspergillus nidulans* reproduces asexually to form conidiophores that produce long chains of spherical uninucleated spores. The asexual life cycle takes ~2-3 days to be completed (Casselton and Zolan, 2002), depending on genetic background, nutrition, and

temperature. *A. nidulans* is a model organism for studies of cell biology and gene regulation in filamentous fungi.

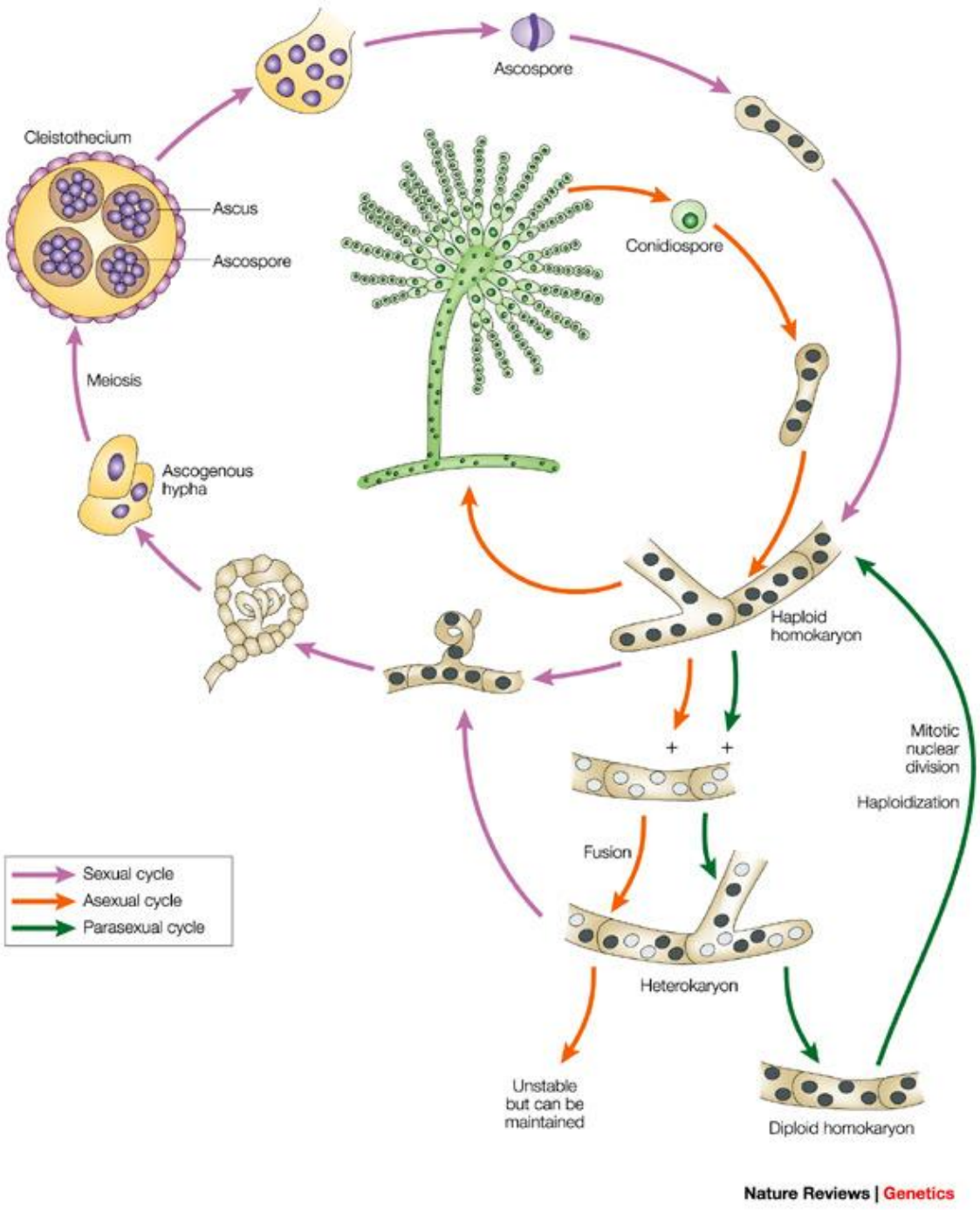

Fig. 5: The life cycle of *Aspergillus nidulans* (adapted from Casselton and Zolan, 2002).

1.8 Objectives

Methionine sulfoxide reductases (MSRs) are important enzymes helping to scavenge reactive species and to repair oxidatively damaged proteins. While the genus *Aspergillus* includes human and plant pathogens as well as beneficial species used to produce foodstuffs and industrial enzymes (Kapoor et al., 1999), the MSRs were never characterized from any *Aspergillus* species.

This project aims at elucidating the role and molecular function of MSR isoforms in *Aspergillaceae,* e.g. the filamentous fungus *Aspergillus nidulans,* which is an established model system for studying development, gene regulation, and metabolic processes. Unlike mammals, *Aspergillaceae* only have one MSRA and one MSRB isoform, specifically reducing the epimeric forms of methionine-S-sulfoxide and methionine-R-sulfoxide, respectively. Hence, *Aspergillaceae* are suited eukaryotic model systems for studying the transcriptional regulation and subcellular organization of MSRs, as well as their role in stress response and cell survival upon oxidizing challenge. This is an important issue because in particular the function of MsrBs is still elusive. Our study characterized MsrA, MsrB and fRMsr through its purification from *A. nidulans* strain TNO2a3. The recombinant enzymes have been expressed, purified and characterized with regards to its substrate specificity, kinetic parameters. The deficiency effects of *msr* gene on the filamentous fungus have been characterized under the physiological and the oxidative stress conditions.

While many studies strongly proved the significant role of Msrs in the cell, only a few target proteins were identified such as ShC/B channels (Ciorba et al., 1997), CaM (Sun et al., 1999), *E. coli* Ffh (Ezraty et al., 2004), and potassium channel hERG1 (Su et al., 2007). In contrary, Methionine-oxidized Apolipoprotein C-II (apoC-II) amyloid fibrils are a poor substrate for hMsrA and hMsrB2 (Binger et al., 2010). Thus the identification of the Msr *in vivo* substrates proteins is essential to determine their involvement in protein regulation and protection against oxidative stress and diseases. Hence, Msrs are strictly specific for methionine sulfoxides reduction, but are they able to repair or protect any bound-MetO regardless to its location among the proteins. The latter is an open question; in order to answer it, we have proposed a hypothesis that might explain which oxidized proteins are expected to be reducible by Msr, the hypothesis focusing on the impact of the neighboring residues next to MetO on the reduction by Msr.

2 Materials and methods

2.1 Construction of fRMsr knockout in *Aspergillus nidulans*

2.1.1 Growth conditions of *A. nidulans*

Aspergillus nidulans strains were cultivated from freshly harvested 10^8 conidia spores/ml according to Pontecorvo et al. (1953) at 37 °C in 50 ml of AMM, containing glucose as the carbon source, $NaNO_3$ as the nitrogen source and the appropriate supplements for each strain, in a 250 ml Erlenmeyer flask. If required, supplements were added as follows: for the strains TNO2a3 (wt) and Δ*fRmsr*; pyridoxine-HCl (0.5 μg/ml), and uracil/uridine (2.2 mg/ml and 1 mg/ml, respectively), for Δ*msrA*; pyridoxine-HCl (0.5 μg/ml) and arginin (5 mM), for Δ*msrB*; uracil/uridine (2.2 mg/ml and 1 mg/ml, respectively) and arginin (5mM).

***A. nidulans* strains**	**Genotype**	**Reference**
TNO2a3 (Δ*nkuA*)	pyroA4 pyrG89 ΔnkuA::argB$^+$	MAM, HKI
Δ*msrA*	ΔmsrA::pyrG$^+$ pyrG89; argB2, pyroA4),	Soriani et al. 2009
Δ*msrB*	pyrG89; argB2, pyroA4, ΔmsrB::pyroA$^+$	Soriani et al. 2009
Δ*fRmsr*	pyroA4 pyrG89 ΔnkuA::argB$^+$	This study

***Aspergillus* minimal medium (AMM) pH 6.5 (NaOH)**	NaNO3	6 g/l
	KH_2PO4	1.52 g/l
	KCl	0.52 g/l
	Agar (Plates)	15 g/l
	After autoclaving the following was added	
	Glucose	1 %
	$MgSO_4$	0.05 %
	Hutner'strace element solution	1 ml/l
	Auxotrophies	
Hutner's trace element solution	$FeSO_4 \times 7H_20$	0.5 g
	$ZnSO_4 \times 7H_2O$	2.2 g
	H_3BO_3	1.1 g
	$CuSO_4 \times 5H_2O$	0.16 g
	$MnCl_2 \times 4H_2O$	0.5 g
	$CoCl_2$-$6H_2O$	0.16 g
	$(NH_4)Mo_7O_{24} \times 4H_2O$	0.11 g
	EDTA	5.0 g
	ddH2O	100 ml
	Sterilized by filtration	

Auxotrophies stock solutions

Arginin	0.5 M	10 ml/l
Pyridoxin-HCl	50 mg/100 ml	1 ml/l
Uridin	1 M	4 ml/l
Uracil	2.2 g/l	

All auxotrophies are sterilized by filtration except Uracil is sterilized by autoclaving

2.1.2 Genomic DNA purification

Genomic DNA of *A. nidulans* was isolated using the MasterPure^{TM} Yeast DNA Purification Kit (Epicentre Biotechnologies, USA). Mycelia were harvested from 16-18 hours old cultures and filtered over miracloth. Approximately 100 mg were transferred to Lysis tube (Analytic Jena, Germany) containing the Cell Lysis solution. The lysed mycelia were incubated for 30-60 min at 65 °C after using the homogenizer at a speed of 5 for 40 sec. Protein Precipitation Reagent (MPC, Epicentre Biotechnologies, USA) was added to the lysed mycelia after 5 min incubation on ice to precipitate the protein. The cellular debris was collected by centrifugation for 10 min at ≥10,000 rpm and the supernatant was transferred to a clean microcentrifuge tube contains isopropanol and mixed thoroughly by inversion. The DNA was then precipitated by centrifugation for 10 min at ≥10,000 rpm and washed with 70% ethanol. The DNA was eluted with ddH_2O (free nucleases) and the DNA concentration was measured using NanoDrop ND-1000 spectrophotometer. The RNase treatment was done using 1 µl of 5 µg/µl RNaseA (Epicentre Biotechnologies, USA).

2.1.3 Deletion cassette

For the purpose of deleting *fRmsr* from *A. nidulans*, a deletion cassette containing 0.76 kb of the 5' which is a product of PCR 1 and 0.74 kb of the 3' a product of PCR 2 untranslated region (UTR) of *fRmsr* was generated. The UTRs were amplified from genomic DNA using specific primers containing flanking *ptrA* sites and the fragments were purified from the gel. The resistance gene pyrithiamine (*ptrA*) for targeted gene replacement (Krappmann et al., 2006) was amplified from PALptrA plasmid using specific primers (PCR 3) and purified from the gel. The deletion cassette was constructed in PCR 4 in which the three fragments (*fRmsr* 5' end (PCR 1), 3' end (PCR 2) and *ptrA* fragment (PCR 3)) were fused together using the frmsr_5` forward primer and the frmsr_3` reverse primer. The entire deletion cassette with a size of 4 kb was obtained by purifying the PCR 4 product from 0.8 % agarose gel and transformed into the Δ*nkuA* strain TNO2a3. Southern blot hybridization analysis was

conducted to confirm homologous recombination of the *ptrA* knockout cassette at the *fRmsr* locus using a PCR amplified 5` UTR as an *fRmsr* probe.

Target	**Sequence**
*fRmsr*_5`_F	5'-CAACGACGGGGATTGATTTG-3'
*fRmsr*_5`_R*(ptrA)*	5'-*GAGGCCATCTAGGCCATCAAG*CGCTTGTGATGCGAGTAGTCG-3'
*fRmsr*_3`_F	5'-GACAGCTTGATTGCTCCTATG-3'
*fRmsr*_3`_R*(ptrA)*	5'-*GGCCTGAGTGGCCATCGAATT*CGGAAGGGAATTGATCTTGGC-3'
*ptrA*_F	5'-GAGGCCATCTAGGCCATCAAGC-3'
*ptrA*_R	5'-GGCCTGAGTGGCCATCGAATTC-3'

2.1.4 Transformation of *A. nidulans*

2.1.4.1 Preparation of protoplasts

The transformation was adapted from the method of Balance and Turner (1985). Mycelia from an overnight culture were obtained by filtration over miracloth, followed by washing with washing solution. These were then incubated in glucanex solution, for at least 3 h, at 28 °C with gentle agitation (80 rpm). When sufficient protoplasts were present, remaining mycelia were removed by filtration using miracloth. The protoplasts were harvested by centrifugation for 10 min at 4,000 rpm at 4 °C. The pellet was washed three times with the washing solution before it was resuspended with the resuspension solution to a final concentration of approximately 5 x 10^7 protoplasts in 150 µl.

2.1.4.2 Protoplast transformation

Protoplasts 75 µl, DNA (1-5 µg) up to 10 µl and 20 µl PEG (polyethylene glycol) solution were added to a 2 ml Eppendorf tube and agitated gently. Subsequently, the tube was incubated on ice for 20 min. At this time, 0.7 ml PEG solution was added to the mixture, and the protoplasts were incubated for further 5 min on ice then 1 ml of resuspension solution was added to the mixture. The protoplasts/DNA mixture was plated by adding it to the top agar (50 ml, 55 °C) followed by pouring onto transformation plates which contained the selective AMM agar. The plates were incubated for 3-5 days at 37 °C.

Glucanex solution	Glucanex (enzyme mix)	0.8 g/20 ml
	Washing solution	20 ml

Washing solution	KCl	0.6 M
	Tris/HCl pH 7.5	0.1 M
	Autoclave sterilization	
Resuspension solution	KCl	0.6 M
	$CaCl_2$	50 mM
	Tris/HCl pH 7.5	10 mM
	Autoclave sterilization	
PEG solution	PEG (8000)	25%
	CaCl2	50 mM
	Tris/HCl pH 7.5	10 mM
	Filter sterilization	
Top agar	AMM (Strain specific auxotrophies)	
	Agar	15 g/l
	Pyrithiamine (selective marker)	0.1 mg
	KCl	0.6 M
Selective medium	AMM (Strain specific auxotrophies)	
	Agar	20 g/l
	Pyrithiamine (selective marker)	0.1 mg
	KCl	0.6 M

2.2 Conformation of the fRMsr knockout

2.2.1 Southern blot analysis

2.2.1.1 DNA extraction and digestion

In order to prove the deletion or ectopic integration of the *ptrA* gene, Southern blot analysis of chromosomal DNA of *A. nidulans* was carried out according to Liebmann *et al.* (2004). Standard techniques in the manipulation of DNA were carried out as described by Sambrook *et al.* (1989). Genomic DNA of *A. nidulans* strain TNO2a3 was prepared from 16-18 hours old cultures using the MasterPure^{TM} Yeast DNA Purification Kit (Epicentre Biotechnologies, USA). DNA was digested for 16 hours at 37 °C using restriction enzymes BamHI from New England Biolabs (Germany) according to the manufacturer's instructions.

2.2.1.2 DNA blotting

The digested DNA was separated on 0.9 % (w/v) agarose gels. The gel was depurinated two times with the depurination solution for 10 min with gentle shaking followed by rinsing with water by which the blue dye was turned into yellow. The DNA was denatured in the gel by soaking the gel in the denaturation solution for 2 times each 15 min with gentle shaking,

followed by brief washing with water. In this last step the dye was turned blue again. The final step of preparing the DNA for the salt transfer in the blotting is to neutralize the gel in the neutralization solution for 2 times each 15 min with gentle shaking. The DNA was transferred onto a Hybond N^+ membrane by overnight capillary blotting followed by crosslinking the DNA to the membrane by UV-crosslinker.

2.2.1.3. Probe preparation

The probe was amplified from strain TNO2a3 gDNA with a specific primer of the 5' end of *frmsr* gene. The amplification was performed using the Phusion High Fidelity DNA polymerase Kit (Finnzymes, Finland). The probe was nonradioactive-labeled during the PCR amplification using the Digoxigenin-11-duTP (Roche Applied Science). The PCR product was run on 0.9 % agarose gel and the band was extracted from the gel using the Zymo Clean Gel extraction Kit (Zymo Research).

2.2.1.4 Hybridization and detection

For detection of UV-crosslinked DNA fragments the DIG High Prime Labelling and Detection System (GE Healthcare Biosciences, Germany) with a non-radioactive labeled DNA probe was used according to the manufacturer's instructions. Blocking and antibody binding was performed in DIG Easy Hyb (GE Healthcare Biosciences). Detection was carried out using CDP Star “ready-to-use” (Roche Diagnostics, Germany) according to the manufacturer’s instructions.

Depurination solution 1x:	0.25 M	HCl (37%)
Denaturation solution 1x:	0.5 M	NaOH
	1.5 M	NaCl
Neutralization solution 1x:	0.5 M	Tris-HCl (pH 7.5)
	1.5 M	NaCl
20x SSC:	3M	NaCl
	0.3M	Na-citrate
		pH 7.0 with HCl
Low Stringency Buffer:	2x	SSC
	0.1%	SDS (filter-sterile)

High stringency buffer:	0.5x	SSC
	0.1%	SDS

2.2.2 *A. nidulans* Msrs expression

2.2.2.1 Isolation of total RNA

For the real-time RT-PCR, $1x10^8$ conidiaspore/ml of *A. nidulans* (TNO2a3) strain, Δ*MsrA*, Δ*MsrB*, and Δ*fRmsr* were used to inoculate 50 ml of AMM in 250-ml flasks that were incubated in a reciprocal shaker (200 rpm) at 37 °C for 14 h. After this period, the germlings were exposed or not to the corresponding stressing agent for 1 h of time at 37 °C and were harvested by centrifugation, washed with distilled water and frozen in liquid nitrogen. Total RNA was extracted using the TRIsure reagent (Bioline GmbH, Germany). Briefly, 50-100 mg of mycelia tissues were homogenized in 1 ml of TRIsure followed by removing the insoluble material by centrifugation at 12,000 xg for 10 min at 2-8 °C and the cleared homogenate was transferred to a fresh tube. The samples were incubated for 5 min at RT before adding 0.2 ml of chloroform per 1 ml of TRIsure used. Cap tubes securely and shake vigorously by hand for 15 seconds followed by incubation for 2-3 min at RT. The sample will separated into a pale green phenol-chloroform phase, an interphase, and a colourless upper aqueous phase that contained the RNA by centrifuge the samples at 12,000 xg for 15 min at 2-8 °C. For precipitating the RNA, the aqueous phase was carefully transferred to a clean tube contained isopropyl alcohol. The samples were incubated for 10 minutes at RT before the centrifugation at 12,000 xg for 10 min. The RNA pellet was washed by 75% ethanol, and then centrifuged at 7500 xg for 5 min at 2-8 °C. The pellet was air-dried for 5-10 minutes then dissolved in DEPC-treated water (BIO-38030) by pipetting the solution up and down, followed by incubation for 10 min at 55-60 °C. The RNA concentration was measured using the NanoDrop ND-1000 spectrophotometer.

2.2.2.2 **Synthesis of the cDNA and the q-real-time PCR**

cDNA of the TNO2a3 strain and the knockouts was synthesized from total RNA using the First Strand cDNA Synthesis Kit Fermentas according to the manufacturer's instructions. Differential gene expression was analyzed by realtime-PCR with the iCycler® Thermal Cycler (Bio-Rad, Munich, Germany). SYBR® Green served as fluorophor for online-monitoring of generated PCR-products. Specific inner primers were synthesized at MWG-Biotech AG (Ebersberg, Germany) to detect the expression of the *A. nidulans msr* genes. The

quantative RT PCR reaction was performed using the Maxima^{R} SYBR Green/ROX qPCR master mix (Fermentas). The data were normalized to *A. nidulans* tublin (TUB).

Target gene	**Sequence**
*tubulin*_For	5'-AGTTCACTGCTATGTTCCGTC-3'
*tubulin*_Rev	5'-TTCATGTTGCTCTCAGCCTC-3'
*msrA*_For	5'-CCCTCTCACACACAACGGAATC-3'
*msrA*_Rev	5'-AATGGCACTGCGATACTGCGTG-3'
*msrB*_For	5'-TGTTTTGAGCCCAGGTAAGCAG-3'
*msrB*_Rev	5'-CGGACTTGAACTTGTGACTCGC-3'
*fRmsr1*_For	5'-TCA ATA ATC GCG ACC GTC TC-3'
*fRmsr1*_Rev	5'-CCG CCA AGA TCA ATT CCC TT-3'
*fRmsr2*_For	5'-GCG GCT TCT GTC GAT TCA GC-3'
*fRmsr2*_Rev	5'-TAT GCG CAG GTC ATT CAG CA-3'

2.2.3 Zone Inhibition assay

Conidia of TNO2a3 (wt strain) and knockouts were harvested from plates and 10^{6} conidiaspores/ml were added to 15-20 ml AMM agar (48 °C) and mixed. The AMM agar was poured in petri dishes and cooled down until it had solidified. In the center of the agar plate, a 1 cm hole was created, which was filled with different H_2O_2, menadione or chloramine-T concentrations. Plates were incubated overnight at 37 °C. The diameter of the inhibition zone was measured (Fig. 5).

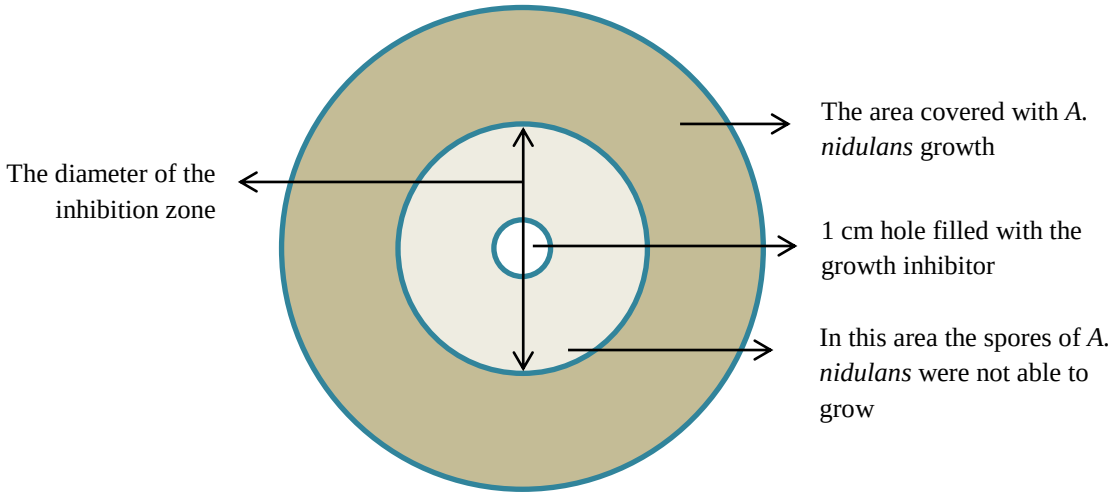

Fig. 5: Diagram shows the inhibition zone assay plate.

2.3 Cloning and expression of Msr proteins in *E. coli*

2.3.1 Comparative genomic analyses of *A. nidulans* Msrs

Human MsrA (NP_036463) and hMsrB2 (NP_036360) proteins sequences were used to identify the *A. nidulans* homologous in the database (NCBI), the BLAST search revealed two putative protein sequence with unknown function. The first putative protein under the accession number (XP_662118.1) comprises the MsrA functional sequence. The second putative protein with the accession number (XP_659536.1) belongs to the MsrBs family. While *E. coli* (NP_416346) and *S. cerevisiae* fRMsr proteins (NP_012854) were used as seed sequences to search for homologous in fungi and only one hypothetical protein was found with accession number (XP_680937).

2.3.2 Cloning of AnMsrs in *E. coli* (XL1 blue)

Total RNA was extracted from *A. nidulans* strain TNO2a3 using the TRIsure reagent (Bioline GmbH, Germany) as mentioned before. cDNA was synthesized using the First Strand cDNA Synthesis Kit Fermentas according to the manufacturer's instructions. The full-length of the coding sequence for *msrA, msrB* and *fRmsr* genes were amplified from TNO2a3 strain's cDNA with specific primers for each gene. For efficient expression and purification in *E. coli* the AnMsrA protein, a truncated version of the gene was also amplified using specific forward primers starting at the second Met. The DNA amplification was performed using the Expand High fidelity PCR Kit (Roche). The PCR products were run on 0.9 % agarose gel and DNA fragments were extracted from the gel using the Zymo Clean Gel extraction Kit (Zymo Research). The *msrA, msrB* and *fRmsr* gel-extracted fragments were first ligated with the pGEM T-vector (pGEM T-vector system I, Promega, USA) using the pGEM and were transformed into the *E. coli* strain XL1 blue. The plasmids were isolated from the positive colonies using the miniprep kit. To confirm the correct sequence for each gene the pGEM-*msrA*, pGEM-*msrB* and pGEM-*fRmsr* plasmids were sequenced at GATC (GATC-biotech, Konstanz, Germany) using T7 primers.

Target gene	**Sequence**
*msrA*_For (**Nde1**)	5'-**CAT**ATGGCATTCGCCGCACCCACC-3'
*msrA*_Rev (**BamH1**)	5'-**GGATCC**TCACTCAGAAAGCGGCGGAAAGC-3'
*msrA*del2_For (**Nde1**)	5'-**CAT**ATGACCTTCTCCACCACTTCTCCG-3'
*msrB*_For (**Nde1**)	5'-**CAT**ATGGCGCCGCCTCCGGTC-3'

*msrB*_Rev (**BamH1**)	5'-**GGATCC**TCACGCTTTAGCCTTCGCACC-3'
*fRmsr*_For (**Nde1**)	5'-**CAT** ATG TCT GGT CGG CCC CAC GC -3'
*fRmsr*_Rev (**BamH1**)	5'-**CAT ATG** TCT GGT CGG CCC CAC GC -3'

The full-length of each *A. nidulans msr* gene was cut out from the plasmid that contains the correct sequence using the restriction enzymes NdeI and BamHI (New England Biolabs). The band with the expected size for each gene was cut out from the gel and extracted using the Zymo Clean Gel extraction Kit (Zymo Research). To produce the AnMsrA, AnMsrB and AnfRMsr as a His-tagged proteins in *E. coli* the DNA fragments were then cloned into the expression vector pET-15b vector (Novagen) using the NdeI and BamHI as cloning sites. The sequences were confirmed by the GATC. The pET-15b harboring the correct insert was transformed into *E. coli* BL21 cells for protein expression.

2.3.3 *E. coli* transformation and plasmid isolation

2.3.3.1 Growth conditions of *E. coli*

E. coli cultures were grown overnight at 37 °C and 180 rpm in 3 ml TY medium (plus appropriate antibiotic) for miniprep, or in 50 ml TY medium for midiprep (plus appropriate antibiotics) or 100 ml for protein purification.

Bacterial strains

XL1 Blue (Stratagene)	For cloning of recombinant DNA and plasmid purification
BL21 (Novagene)	For protein expression and purification of *A. nidulans* Msrs
M15 (Qiagen)	For protein expression and purification of human Msrs

Bacterial TY medium	1 %	Bacto-Trypton
	0.5 %	yeast extract
	0.5 %	NaCl
	1.5 %	Agar (Plates)
	Autoclave sterilizing	

2.3.3.2 Transformation

E. coli strains XL1 blue, M15 or BL21 were transformed according to the $CaCl_2$ method described by Sambrook *et al.*, (1989).

2.3.3.3 Plasmid isolation

Miniprep isolation of plasmid DNA from *E. coli* was performed by alkaline lysis, according to Birnboim and Doly (1979). For plasmid DNA isolation, the MiniPrep Plasmid DNA purification Kit (Promega, USA) was used according to the manufacturer's instructions. Midiprep of plasmid DNA was done using MidiPrep Plasmid DNA purification Kit (Promega, USA) according to the manufacturer's instructions.

2.3.4 Expression and purification of recombinant AnMsr proteins in *E. coli*

The *E. coli* strains BL21 harboring pET-15b-msrA, pET-15b-msrB or pET-15b-fRMsr were grown in TY medium containing until OD_{600} reached 0.4-0.6 or in the Overnight Express™ instant TB medium (Novagen, Germany). MSR proteins expression was induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37 °C for 3 hours or for overnight at 28 °C. The cells were then harvested by centrifugation at 5,000 xg for 10 min. 1 mM (IPTG). The cell pellet was dissolved directly in the lysis buffer; 1 mg lysozyme was added to the cell suspension and was incubated for 30-60 min at 4 °C with gentle shaking. After the incubation the cells were sonicated for 40 sec. The lysed cells were centrifuged at 11,000 xg for 15 min at 4 °C and the clear supernatant was incubated for 60 min at 4 °C with Ni-NTA agarose (Promega, Germany) for binding the His-tagged proteins to Ni-NTA beads. The Ni-NTA beads with the His-tagged protein were washed with washing solution A then with washing solution B, one time each. The His-tagged proteins were eluted from the Ni beads with the elution solution. The eluted protein was then washed three times with Tris/HCl (pH 7,5) and the Amicon Ultra-4 membrane filter tube 10 kD was used to reconcentrate the protein and to remove the undesired protein.

Lysis buffer (pH 8.0)	5 mM Imidazol 50 mM NaH_2PO_4 300 mM NaCl
Washing solution A (pH 8.0)	10 mM Imidazol 50 mM NaH_2PO_4 300 mM NaCl
Washing solution B (pH 8.0)	20 mM Imidazol 50 mM NaH_2PO_4 300 mM NaCl
Elution solution (pH 8.0)	250 mM Imidazol 50 mM NaH_2PO_4 300 mM NaCl

2.3.5 Protein concentration and SDS-PAGE

Protein concentration was determined according to Bradford (1976) using the CoomassiePlus™ Protein Assay reagent (Thermo Scientific).Protein samples were dissolved in sample loading buffer and heated at 95°C for 5 min and then separated using 12 % acrylamide gel. The gel was stained with Coomassie blue to confirm the purity of the recombinant proteins.

Sample loading buffer	TrisHCl pH 6.8 70 mM β-mercaptoethanol 5 % Glycerol 40 % SDS 3 % bromophenyl blue 0.05 %
Coomassie blue staining	Coomassie R250 (for gels) 2 g/l Acetic acid 10 %

2.4 Enzymatic activity of Msrs

2.4.1 Purification of hMsrA and hMsrBs

Human MSRs were cloned before in the lab, hMsrA (Kuschel et al., 1999), hMsrB2 (Jung et al., 2002) and hMsrB3 (Hansel et. al., 2003). Human MsrA, MsrB2 and MsrB3 were cloned into the expression vector pQE30 (Qiagen) and then transformed into the *E.coli* M15 strain. The proteins were purified as performed for *A. nidulans* MSRs proteins (see section 3.4.).

2.4.2 Enzymatic activity of *A. nidulans* MsrA and MsrB

The enzymatic activity for AnMsrA, AnMsrB, AnfRMsr, hMsrA hMsrB2 and hMsrB3 were determined using the reverse-phase HPLC (SOURCE 15RPC ST 4.6/100, GE Healthcare, Germany) column and synthetic pre-oxidized peptide as a substrate. The oxidized peptide was synthesized at Peptide-Specialty-Laboratories GmbH (Heidelberg, Germany) with a sequence KIFM(O)K-Dnp (MW 933). The 2,4-diNitroPhenol (Dnp) is a chromophore used as derivatization agent added to the C-terminal of the peptide for monitoring the reaction results on the HPLC. The reduced form was separated from the oxidized form at room temperature at a flow rate of 1.5 ml/min using a gradient of 1-100 % with solvent (A) containing degassed filtered H_2O with 0.1 TFA and solvent (B) containing degassed acetonitrile with 0.1 TFA for 20 min. Detection of Met derivatives was visualized at wavelength 365 nm. The enzymatic reaction was performed in 50 mM Tris/HCl (pH 8.0) buffer, 20 mM DTT, 5 µg MSR and the

peptide, the incubation was at 37 °C for 30 min. The value of peak area was determined using the UNICORN 3.21 software and the reduction percentage was calculated from average of three reactions using the following equation:

$$\textbf{The Reduction percentage} = \left(\frac{\textbf{MetO}_{\textbf{red}}}{(\textbf{MetO}_{\textbf{red}} + \textbf{MetO}_{\textbf{ox}})/2}\right)*100$$

Where $MetO_{red}$ is the value of the area under reduced form peak, and $MetO_{ox}$ is the value of the area under oxidized form peak.

2.4.3 Enzymatic activity of *A. nidulans* fRMsr

The activity of AnfRMsr was determined by a coupled assay. The reaction mixture of total 200 µl contained 5 µg of purified recombinant anfRMsr, 5 µg *A. nidulans* thioredoxin (Trx) (Thön et. al., 2007), 0.5 µl *A. nidulans* thioredoxin reductase (TrxR) (Thön et. al., 2007), 0.4 mM NADPH and 1 mM free MetO. The reaction was allowed to take place at 37 °C for 15 min, and NADPH oxidation was analyzed immediately at OD 340. The activity was calculated using NADPH extinction coefficient (6220 M-1cm-1). This assay was also used to determine the kinetic values for all the MSR enzymes from human and from *A. nidulans* and the data were analyzed using the Igor pro software.

2.4.4 Specificity of *A. nidulans* MSR using capillary electrophoresis (CE)

The free methionine sulfoxide specificity of AnMsr enzymes and the ability of these enzymes to use the free MetO as a substrate were determined using the capillary electrophoresis (CE). CE experiments were performed on a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Krefeld, Germany) equipped with a UV-Vis diode array detector using a 50/60.2 cm, 75 µm id, 375 µm od fused-silica capillary (BGB Analytik Vertrieb, Schloßböckelheim, Germany). All procedures were carried out at 20 °C. The derivatization of the methionine in the reaction with dabsyl chloride was described previously by (Zhu et al., 2013). Briefly, 100 µl of standard solutions containing 1 mM Met, 3 mM Met(O) and 0.5 mM β-Ala as internal standard in 150 mM sodium bicarbonate buffer, pH 9, were mixed in a 1 ml Reacti Vial (Fischer Scientific, Schwerte, Germany) with 200 µl of a 8.5 mM solution of dabsyl chloride in acetone. The mixture was heated at 70 °C for 15 min. Subsequently, the vials were put in ice for 5 min. The organic solvent was removed by a gentle stream of nitrogen at RT, the residue was frozen at –80 °C and lyophilized. The residue was then

reconstituted in 250 µl 20% (v/v) aqueous methanol followed by centrifugation at 5000 rpm for 15 min. The supernatant was analyzed or stored at –20 °C. Samples for method validation and the Msr incubations were diluted with an equal volume of a 1 mM solution of β-Ala in 150 mM sodium bicarbonate buffer, pH 9.0. The mixture was vortexed and 100 µl were derivatized as described above for the standard solutions.

2.4.5 Mutagenesis of the *A. nidulans msr* genes

The QuikChange II Site-Directed Mutagenesis Kit (Agilent technologies, USA) was used to substitute the cysteine to serine. The cysteine positions were predicted by sequence alignment with different organisms. For AnMsrA the Cys at positions 52, 78, 91, and 200 were substituted individually to Ser. In AnMsrB, three Cys at positions 123, 101 and 53 were replaced by Ser, whereas for AnfRMsr Ser was replaced the Cys at positions 104, 114 and 138. Briefly, with the DNA template was pET-15b carrying AnMsrA, AnMsrB or AnfRmsr and the specific primers were designed with the point mutation which needs to be inserted in the middle of their sequence. The PCR reactions were performed as recommended in the kit instructions, after PCR reactions were done; wild-type template was digested with DpnI for 1 hour at 37 °C. The PCR products were transformed into *E. coli* XL1 Blue cells. Mutations were confirmed by DNA sequencing at GATC (GATC Biotech, Germany). Plasmids carrying correct mutations were transformed into *E. coli* BL21 (DE3) and protein expression was carried out as described above (section 3.4.). The ability of the mutated MSRs proteins to reduce MetO were determined using two methods as mentioned in sections 4.2 and 4.3.

This technique was also used to substitute the Asp134 and Glu99 in AnMsrA to different amino acids and the Arg141 in AnMsrB to Asp or Asn. The template DNA for all the AnMsrA mutations was pET-15b-*msrA* except for the double mutants was pET-15-*msrA*-DD to create the reverse mutant *msrA*-DE and pET-15b-*msrA*-QD to create neutral mutant *msrA*-QN.

Target gene	**Sequence**	
AnMsrA		
QD (E99Q)	For.	5'-GGTGATTTTAAGGGCCTGAGCATGCCCAGTATC-3'
	Rev.	5'-GATACTGGGCATGCTCAGGCCCTTAAAATCACC-3'
DD (E99D)	For.	5'-GGTGATTTTAAGGGCGTCAGCATGCCCAGTATC-3'
	Rev.	5'-GATACTGGGCATGCTGACGCCCTTAAAATCACC-3'
EN (D134N)	For.	5'-CGATACTGCGTGCCAACGTTCGGGCCCTGTTGGTTC-3'

	Rev.	5'-GAACCAACAGGGCCCGAACGTTGGCACGCAGTATCG-3'
EE (D134E)	For.	5'-GCGATACTGCGTGCCAACCTCCGGGCCCTGTTGG-3'
	Rev.	5'-CCAACAGGGCCCGGAGGTTGGCACGCAGTATCGC-3'
DE (E99D+D134E)	For.	5'-GCGATACTGCGTGCCAACCTCCGGGCCCTGTTGG-3'
	Rev.	5'-CCAACAGGGCCCGGAGGTTGGCACGCAGTATCGC-3'
QN (E99Q+D134N)	For.	5'-CGATACTGCGTGCCAACGTTCGGGCCCTGTTGGTTC-3'
	Rev.	5'-GAACCAACAGGGCCCGAACGTTGGCACGCAGTATCG-3'
RD (E99R)	For.	5'-GGTGATTTTAAGGGCCCGAGCATGCCCAGTATC-3'
	Rev.	5'-GATACTGGGCATGCTCGGGCCCTTAAAATCACC-3'
ER (D134R)	For.	5'-GATACTGCGTGCCAACGCGCGGGCCCTGTTGGTTC-3'
	Rev.	5'-GAACCAACAGGGCCCGCGCGTTGGCACGCAGTATC-3'
msrA-Cys52Ser	For.	5'-CAACACCCCAAAAAGACCCTGCGGCG-3'
	Rev.	5'-CGCCGCAGGGTCTTTTTGGGGTGTTG-3'
msrA-Cys78Ser	For.	5'-GCTGTGTTTCCTCCGGAATATCCAACCTTTGC-3'
	Rev.	5'-GCAAAGGTTGGATATTCCGGAGGAAACACAGC-3'
msrA-Cys91Ser	For.	5'-CAGTATCTCCCGTAGAAACAGCCCGAT-3'
	Rev.	5'-ATCGGGCTGTTTCTACGGGAGATACTG-3'
msrA-Cys200Ser	For.	5'-CAAAGTGTGCAGGAGACTCATATCCAGATGG-3'
	Rev.	5'-CCATCTGGATATGAGTCTCCTGCACACTTTG-3'
AnMsrB		
Arg121Asn	For.	5'-CTGTTCACACAATGGTTCTCATCCGTTGGCG-3'
	Rev.	5'-CGCCAACGGATGAGAACCATTGTGTGAACAG-3'
Arg121Asp	For.	5'-CTGTTCACACAATGGTCCTCATCCGTTGGCG-3'
	Rev.	5'-CGCCAACGGATGAGGACCATTGTGTGAACAG-3'
msrB-Cys123Ser	For.	5'-CTGTTCACAGAATGTCTCTCATCCGTTG-3'
	Rev.	5'-CAACGGATGAGAGACATTCTGTGAACAG-3'
msrB-Cys53Ser	For.	5'-TATAGCGGTGCGTGGGAGCCCGCGCAG-3'
	Rev.	5'-CTGCGCGGGCTCCCACGCACCGCTATAC-3'
msrB-Cys101Ser	For.	5'- CGCAGTTCGTAGAAGTGATCTCC-3'
	Rev.	5'- GGAGATCACTTCTACGAACTGCG-3'
AnfRMsr		
*frmsr-C*ys104Ser	For.	5'-GGGCCGTCCAGCTAGCCAGGAGATCCGG-3'
	Rev.	5'-CCGGATCTCCTGGCTAGCTGGACGGCCC-3'
frmsr-Cys114Ser	For.	5'-TTGGTCGCGGCGTGAGCGGGGCGGCGGCG-3'
	Rev.	5'-CGCCGCCGCCCCGCTCACGCCGCGACCAA-3'
frmsr-Cys138Ser	For.	5'-GGGCACATTGCTTCTGATGCGAGTAGTCG-3'
	Rev.	5'-CGACTACTCGCATCAGAAGCAATGTGCCC-3'

2.5 Msrs activity and peptide specificity

2.5.1 Peptides sequences

A set of synthetic pre-oxidized peptides were used to determine the substrate specificity of both *A. nidulans* and human MsrAs and MsrBs. The peptides were synthesized at Peptide-Specialty-Laboratories GmbH (Heidelberg, Germany).

Peptide code	Peptide sequence	Mass	Description
KIFm	K-I-F-M(O)-K-Dnp	932.4	Standard peptide for the enzymatic activity
mR	K-G-T-V-M(O)-R-S-L-K-Dnp	1285.66	
mV	K-G-T-V-M(O)-V-S-L-K-Dnp	1228.6	Based on peptide GTVMRSL (Sun et al., 1999)
mD	K-G-T-V-M(O)-D-S-L-K-Dnp	1244.59	
DmD	V-D-M(O)-D-Dnp	745.2	The influence of the acidic amino acids
NmN	K-N-M(O)-N-K-Dnp	900.399	Addressing the influence of flanking Lys residues
NmN	N-M(O)-N-Dnp	628.2	
FmF	K-F-M(O)-F-K-Dnp	966.45	Hydrophobic or aromatic nature of MetO flanking amino acids
VmV	K-V-M(O)-V-K-Dnp	870.45	
KmK	K-K-M(O)-K-K-Dnp	928.5	Positive or negative charges flanking MetO on both sides
EmE	K-E-M(O)-E-K-Dnp	930.399	
DmD	K-D-M(O)-D-K-Dnp	902.36	
DmN	K-D-M(O)-N-K-Dnp	901.38	Unilateral distribution of flanking charged amino acids
NmD	K-N-M(O)-D-K-Dnp	901.38	
KmF	K-K-M(O)-F-K-Dnp	947.477	
FmK	K-F-M(O)-K-K-Dnp	947.477	
KmE	K-K-M(O)-E-K-Dnp	929.45	
EmK	K-E-M(O)-K-K-Dnp	929.45	

2.5.2 HPLC Assay and Data analysis

The activity of the MSRs was determined as mentioned in section 4.2. The reactions with the wild-type enzymes were performed using 2µg from each enzyme and 10 µg for the mutated enzymes. The data were collected from three independent reactions and the average was used to calculate the reduction percentage, and standard deviations of errors were calculated.

3 Results

3.1 *Aspergillus nidulans msr* genes in the database

Many previous studies showed the significant role for Msr enzymes in different cell types. Based on the substrate specificity, Msr enzymes exist in three types: MsrA, MsrB and fRMsr. The number of isoforms of each Msr type is widely different among the different organisms, however, there are at least one MsrA and MsrB in each organism (Kaufman et al., 2005). While *A. nidulans* can be considered as a key fungal model system for genetics and cell biology, MSRs were never characterized from any *Aspergillus* species. This organism provides many advantages over many organisms such as, in contrast to mammals and plants *A. nidulans* only have one isoform of each Msr type, also it is a multicellular organism which offers a more complicated system than bacteria and yeast. In order to investigate the role and the molecular function of all three Msr enzymes from one organism, we analyzed the *A. nidulans* genome for Msr homologos.

3.1.1 Methionine sulfoxide reductase A

A database record using human MsrA protein as a template revealed only one candidate for MsrA in *A. nidulans* under the accession number (XP_662118.1). This sequence shows 64 % conserved sequence with yeast (identity 44 %), 57 % with *A. thaliana* (identity 37 %), 52 % with *E. coli* (identity 37 %) and 50 % with human MsrA (identity 33 %) (Fig. 6A). The sequence alignment confirms that the hypothetical protein has the signature MsrA sequence GCFWG, which contains the catalytic Cys (CysA52 *A. nidulans* MsrA) for MsrA (Hansel et al., 2002). In contrast to hMsrA where the recovery cysteines (CysB and CysC) are separated by a glycine rich region, the C-terminus in *A. nidulans* protein has one Cys and lacks the glycine-rich region, suggesting that MsrA of the fungus might be using a different catalytic mechanism for Met-S-O reduction. Analysis of the *Aspergillus* genome database showed that there is a single candidate for the MsrA gene in some of *Aspergillus* species such as *A. niger*, *A. terreus*, *A. flavus, A. clavatus*, *A. orzyae*, *A. fumigatus* and *N. fischeri*. The similarity of *A. nidulans* MsrA candidate protein is high among the *Aspergillus* genus. For example there are 94 % (identity 84 %) shares with the human pathogen *A. fumigatus*. This suggests that characterization MsrA in *A. nidulans,* which is a safer organism, will enrich our knowledge about the role of Msr enzymes in such organisms. Figure (6B) shows the database MsrA from *A. nidulans* lacking the mitochondrial targeting sequence (MTS) at the DNA and protein level. While the MTS for human MsrA is 23 amino acids, it is expected to be a 37 amino acids at the N-terminus of the AnMsrA protein.

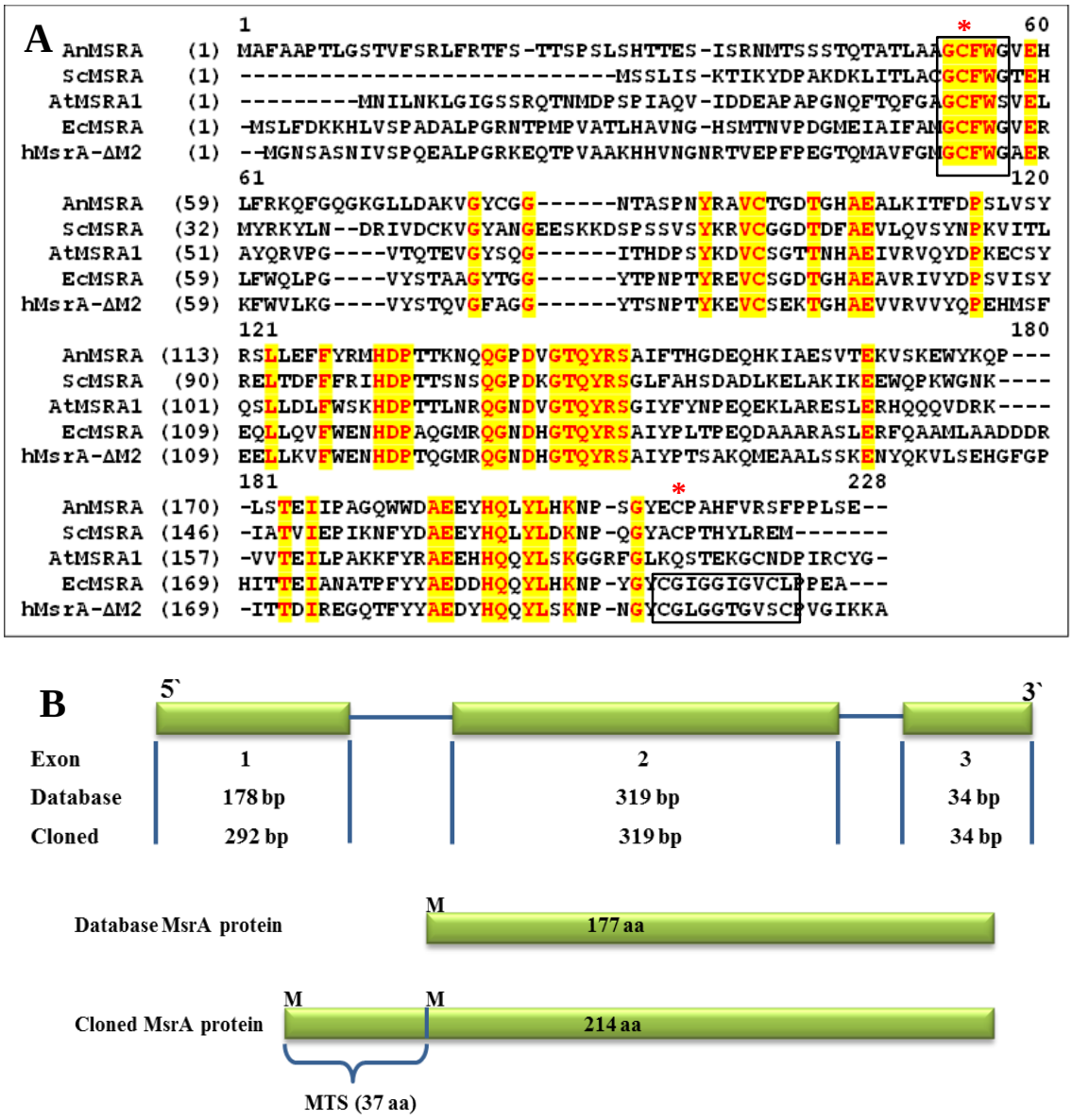

Fig. 6: *A. nidulans* methionine sulfoxide reductase A. (A) Protein sequence alignment. Conserved residues are highlighted. The predicted functional Cys (C) residues are indicated with asterisks. AnMSRA: from *Aspergillus nidulans*; ScMSRA: *Saccharomyces cerevisiae*; AtMSRA: *Arabidopsis thaliana*; EcMSRA: *Escherichia coli*; hMSRA: truncated human. **(B)** Gene organization, exon and protein length are shown for *A. nidulans* MsrA from NCBI database and the cloned sequence.

3.1.2 Methionine sulfoxide reductase B

A BLAST search using hMsrB2 revealed one putative sequence in *A. nidulans* with accession number XP_6595336.1 (NCBI database). This protein sequence showed 62 % conserved sequence with yeast (identity 50 %), 56 % with both of *A. thaliana* (identity 42 %) and with hMsrB3 (identity 40 %) (Fig. 7A). The alignment confirms that the *A. nidulans* MsrB sequence shares the zinc-binding motif (CxxC) with the other MsrBs at positions 50-53 and 98-101 (from *A. nidulans* MsrB). While hMsrB1 is known as a selenoprotein and the only MsrB contains a selenoCys instead of the normal Cys as the catalytic Cys, the data showed

that *A. nidulans* MsrB has a normal Cys. Interestingly, the BLAST search using *A. nidulans* MsrB in the *Aspergillus* genome database revealed that there is a single candidate for MsrB in each species except for *A. oryzae*, which does not contain a homologous sequence of MsrB.

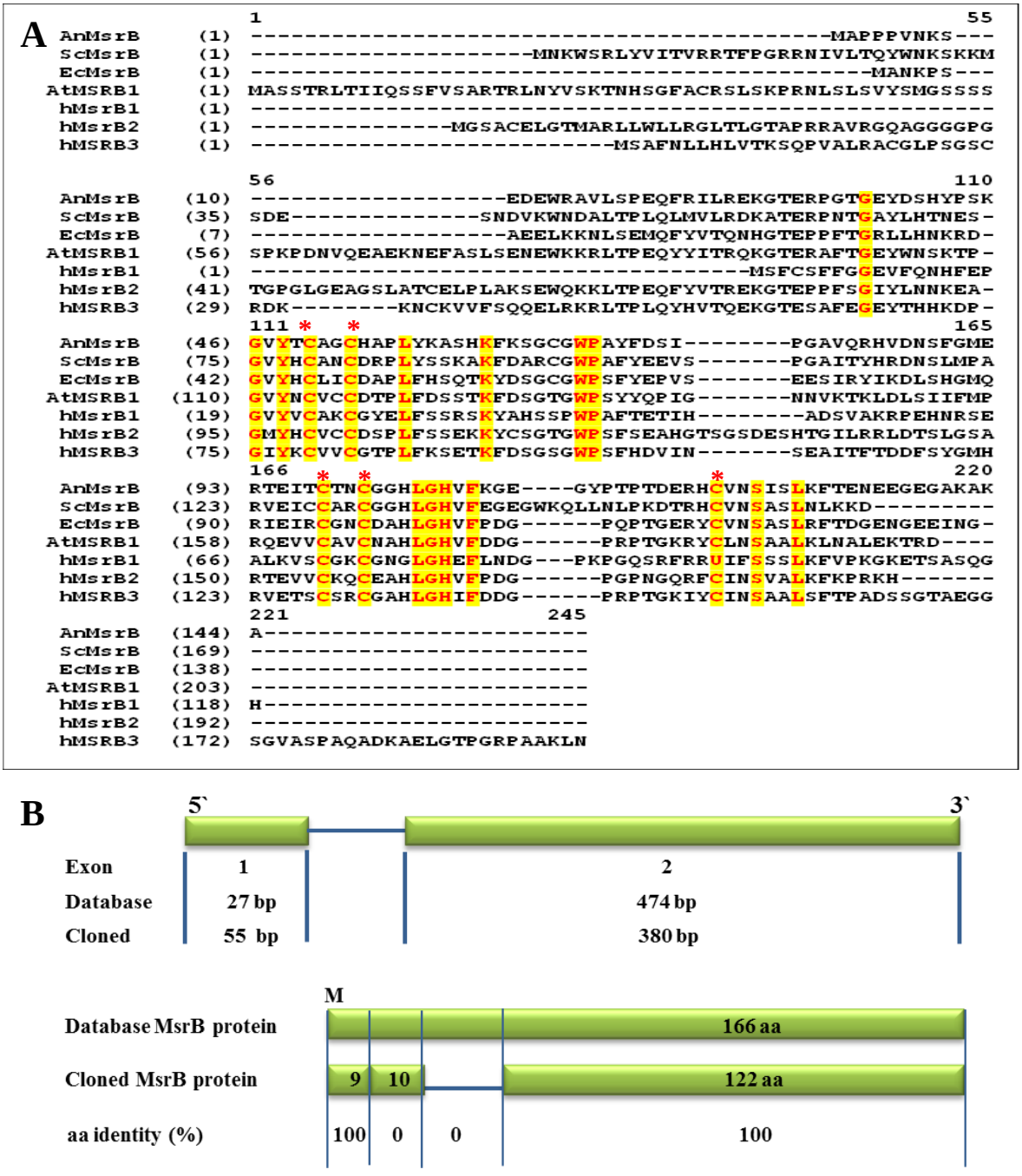

Fig. 7: *A. nidulans* methionine sulfoxide reductase B. (A) Protein sequence alignment. Conserved residues are highlighted. The predicted functional Cys (C) residues are indicated with asterisks. AnMSRB: from *Aspergillus nidulans*; ScMSRB: *Saccharomyces cerevisiae*; AtMSRB: *Arabidopsis thaliana*; EcMSRB: *Escherichia coli*; hMSRB1/2/3: human. **(B)** Gene organization, exon and protein length are shown for *A. nidulans* MsrB from NCBI database and the cloned sequence.

This suggests that *A. oryzae* might be using an unknown strategy for the reduction of methionine sulfoxide. The NCBI database sequence for *A. nidulans* MsrB shows that this gene has two exons (27 bp and 474 bp) and resulted in a 166-aa protein (Fig. 7B). However, our results show (the cloning details at section 3.3) that the cloned MsrB also consists of two exons but with different lengths (55 and 380 bp). As a result of this difference, the recombinant protein is missing 21 amino acids and 10 amino acids differ from the database protein from Glu10 to Pro19.

3.1.3 Free Methionine R-sulfoxide reductase

Recently a third member of MSR family was identified in *E. coli* (Lin et al., 2007) and yeast (Le et al., 2009); the authors stated that it is limited to unicellular organisms. Their finding answered the question how the R-epimer of the MetO can be reduced in lower organisms. Interestingly, the BLAST search using yeast fRMsr (*S. cerevisiae*) revealed a single sequence with accession number XP_680937 in *A. nidulans*. The fRMsr protein from *A. nidulans* shares 72 % conserved sequence with yeast (identity 53 %) and 57 % with *E. coli* fRMsr (identity 37 %) (Fig. 8A). We also used this sequence in the *Aspergillus* genome database in order to investigate the abundance of fRMsr in the Aspergillus genome.

The data confirmed the presence of a single candidate for fRMsr in all of the *Aspergillus* species tested. The similarity with the others *Aspergillus* genus members is as follows; 88 % with *N. fischeri*, *A. flavus* and *A. terreus*; 87 % with *A. oryzae* and *A. niger* while 85 % with *A. clavatus* and only 64 % with the human pathogenic species *A. fumigatus*. The AnfRMsr gene consists of 4 exons; the alignment of the database and the cloned sequence shows that exon 1 and 4 are identical while exon 2 and 4 are shorter in the cloned sequence by a single bp and 20 bp, respectively. Accordingly, the cloned AnfRMsr protein is shorter and misses 7 amino acids between Val39 and Ser40 (Fig. 8B).

3.2 Msr knockouts in *A. nidulans*

The alignment showed that the filamentous fungus *A. nidulans* has a single gene for each type of methionine sulfoxide reductases. Thus *A. nidulans* was chosen to be a model for a multicellular organism for studying the MSRs. While we were preparing for the project, the Δ*msrA* and Δ*msrB* knockouts were already created and characterized by Soriani et al., 2009. These two single knock outs were kindly provided by Prof. Gustavo H. Goldman (Soriani et al., 2009). We created the third knock out (Δ*fRmsr*) from the wild-type strain TNO2a3 using the PCR-based deletion strategy.

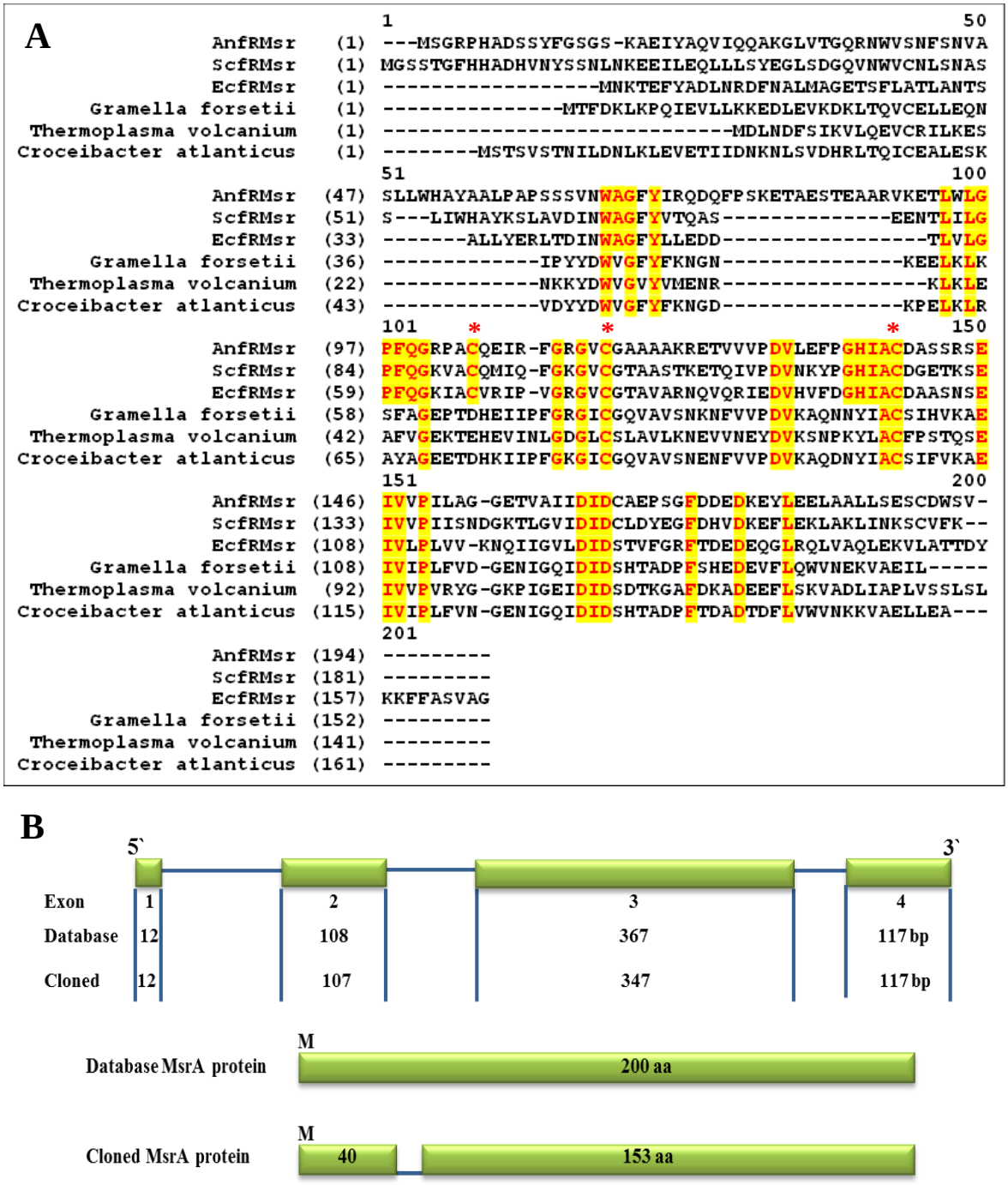

Fig. 8: *A. nidulans* free R-methionine sulfoxide reductase. (A) Protein sequence alignment. Conserved residues are highlighted. The predicted functional Cys (C) residues are indicated with asterisks. AnfRMsr: from *Aspergillus nidulans*; ScfRMsr: *Saccharomyces cerevisiae*; EcfRMsr: *Escherichia coli*. **(B)** Gene organization, exon and protein length are shown for *A. nidulans* MsrB from NCBI database and the cloned sequence.

The *fRmsr* gene was deleted in the TNO2a3 background using a pyrithiamine resistance cassette. A DNA fragment fusion PCR technique was employed wherein primers frmsr_5' _F and frmsr_5'_R were used to amplify the 1 kb upstream sequence of *fRmsr* gene. Similarly, fRmsr_ 3'_F and fRmsr_3'_R primers amplified a 1 kb downstream region of the *fRmsr* gene. Oligonucleotides ptrA_F and ptrA_Rev were used to amplify the pyrithiamine resistance cassette from plasmid PALptrA. The beginning of fRmsr_5'_R (*ptrA*) primer contained a sequence complementary to the sequence of ptrA_for and frmsr_3'_Rev (*ptrA*) primers also harbored a complementary sequences to ptrA_R primers. This ensured that the pyrithiamine gene fused in the middle of the up- and downstream sequence of *fRmsr* gene. In the first round of PCR, the up- and downstream sequences of the *fRmsr* gene and the pyrithiamine (*ptrA*) gene were amplified separately. To fuse the flanking regions of the *fRmsr* gene with the pyrithiamine gene, the PCR products for all were purified and fusion PCR was performed. For this final PCR, primers annealing to the beginning of upstream (fRmsr_5'_F) and to end part of downstream (fRmsr_3'_R) sequences were used (Fig. 9). Thus, the final PCR mix for deletion of *fRmsr* constituted of its purified 5' and 3' flanking region, the purified *pyrithiamine* gene and the above mentioned primer pairs.

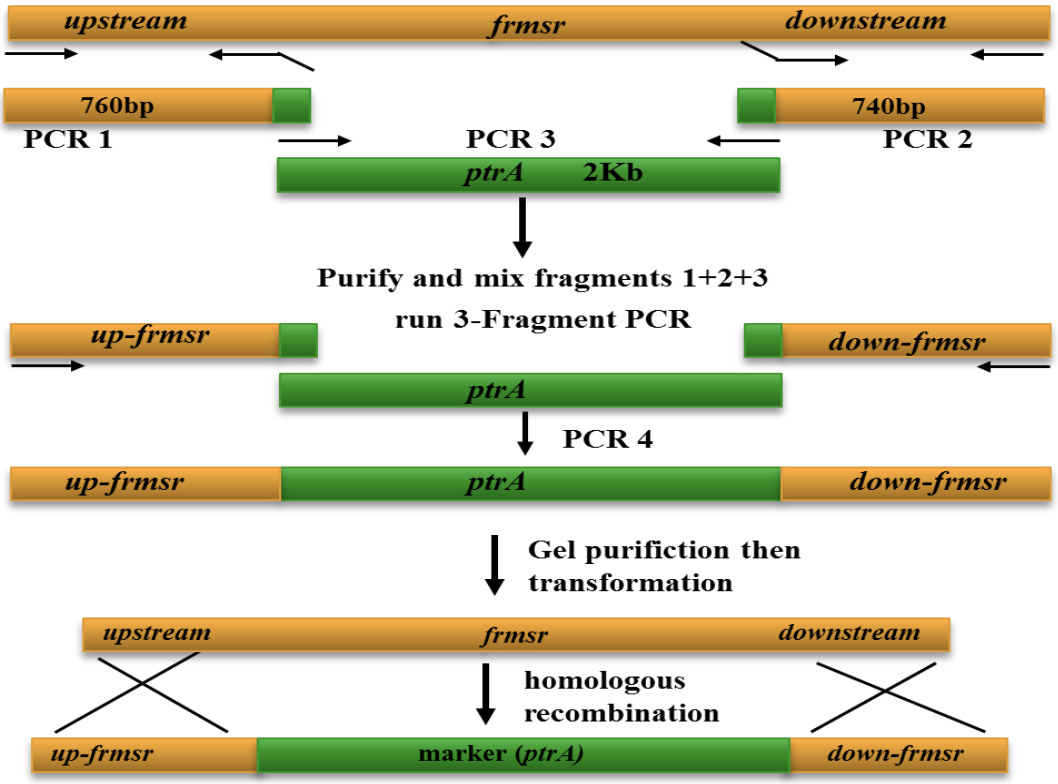

Fig. 9. Cartoon illustration of the PCR-based gene deletion strategy in *Aspergillus*. A deletion cassette contains *pyrithiamine* (*ptrA*) gene was used to knock out *fRmsr* gene in *A. nidulans* TNO2a3 strain.

Transformation of *A. nidulans* TNO2a3 protoplasts with the final PCR products to generate the *fRmsr*-deletion strain was carried out and several transformants were selected by their ability to grow on a selective medium (see Material and Methods for more details). The knockout was analyzed by quantitative real-time PCR and Southern blot analysis as shown in Fig. (10). A 760 bp probe from the 5' region was used for the southern blot analysis and the data established that the *fRmsr* was positively replaced by pyrithiamine. The real-time PCR analysis was successfully used to distinguish between the knockouts candidates as also showed in Fig. (10B). The lack of *fRmsr* mRNA in some knockout candidates (i.e. lanes 1, 3 and 5-7 in Fig. 10B) confirmed the success of *fRmsr* deletion in the TNO2a3 strain.

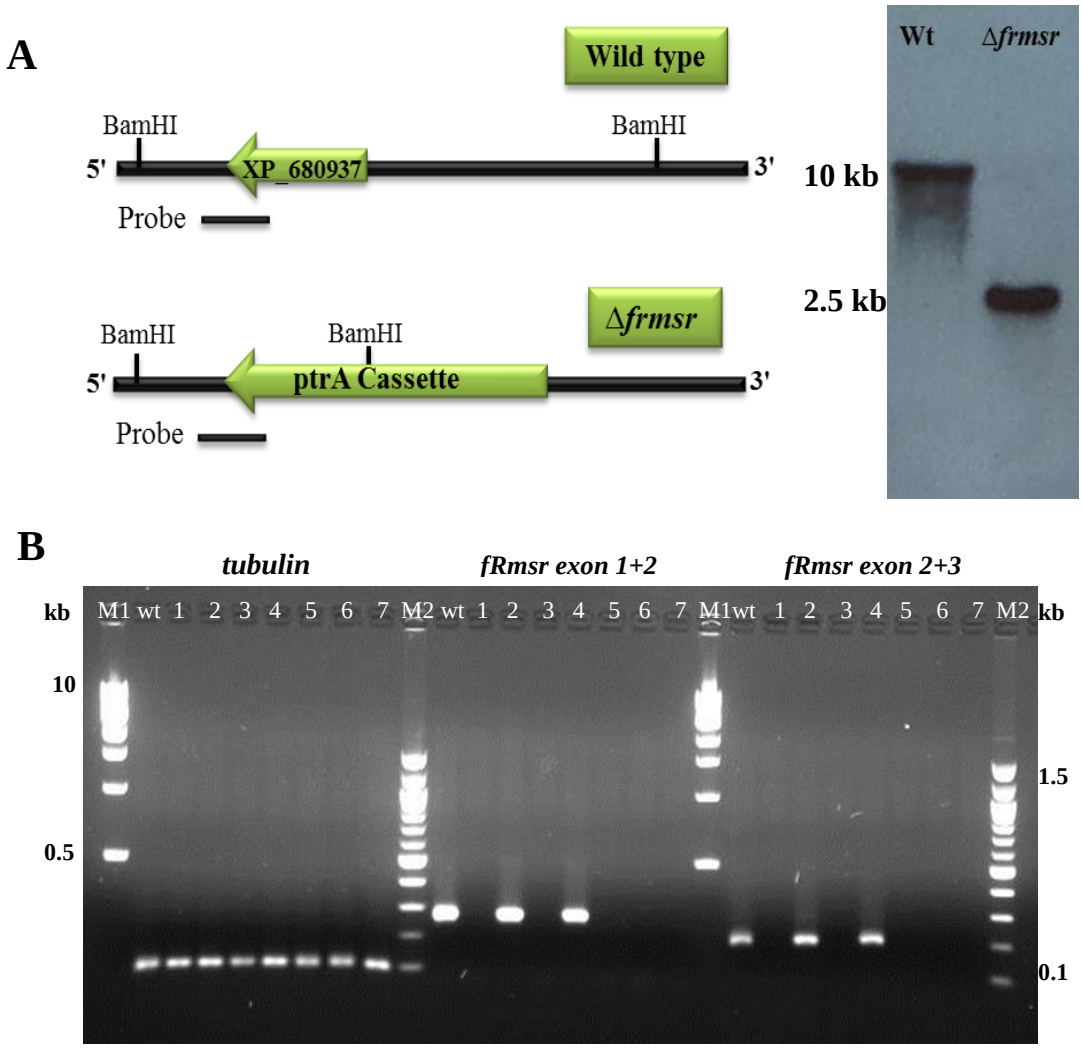

Fig. 10: Genome structure and Southern blot analysis of the Δ*fRmsr* knockout. (A) Southern blot analysis of the Δ*fRmsr* knockout was performed by digesting genomic DNA with *BamHI*. The detection showed a band with a size of 10 kb for the wild-type strain and a band of 2.5 kb for the Δ*fRmsr* strain. **(B)** Confirmation of *fRmsr* deletion in *A. nidulans* TNO2a3. Two different primers were used to detect *fRmsr* gene expression in the knockout candidates in comparison with the expression of *tubulin* as housekeeping gene. M1: 10 kb ladder and M2: 0.1 kb ladder (BioLabs); wt: wild-type strain; lanes 1-7: knock out candidates.

3.2.1 Characterization of *msr* knockouts

We successfully identified three genes for Msr, one for each type and two single knockouts were provided by collaboration with Prof. Gustav Goldman and the third single knockout was created during this study. Southern blot analysis and real-time PCR technique were used successfully to confirm the elimination of the *msr* genes. In this section we will focus on the characterization of these single knockouts on the morphological, physiological and molecular levels.

3.2.1.1 Morphology analysis

The morphology of Δ*msrA,* Δ*msrB* and Δ*fRmsr* knockouts was compared on AMM agar plates incubated at 37 °C for 3 days. The knockouts showed no obvious difference in the morphology of the knockouts compared to the wild type. While the spores were greenish for the wild type and Δ*msrA* and Δ*fRmsr*, the spore color was more brownish for Δ*msrB* (Fig. 11). Thus, MSR enzymes appear not to be required for fungal viability and growth under normal conditions, but lack of MsrB affected the spore color.

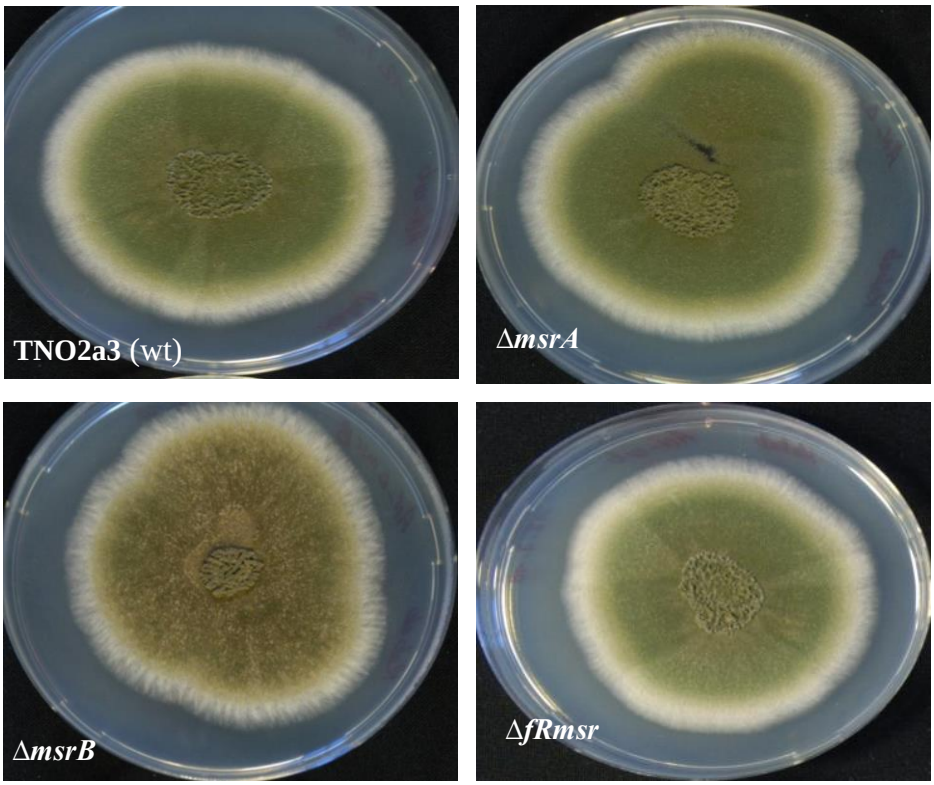

Fig. 11: Morphology analysis of the Δ*msrA,* Δ*msrB and* Δ*fRmsr* knockouts strains in comparison with the wild-type strain (TNO2a3). 10^4 conidiaspore were point inoculated on AMM agar plates and incubated at 37 °C for 3 days.

3.2.1.2 Radial growth rate

The radial growth rates were determined at 37 °C on AMM agar plates. Drops of the conidial inocula with identical starting concentrations where seeded and the radial growth diameter of the cultures was determined over a period of 120 hours. The Δ*msrB* strain showed consistently delayed growth during the first 24 h (Fig. 12A) while the wild-type, Δ*fRmsr* and Δ*msrA*, did not show differences in growth during the inspection period. Following the initial lag phase of growth, the Δ*msrB* strain had the same growth rate as a wild type and the other mutant (Fig. 12B, 48 h – 120 h). This initial delay in growth of Δ*msrB* suggests that MsrB might be critically involved in early steps of fungal growth on solid substrates.

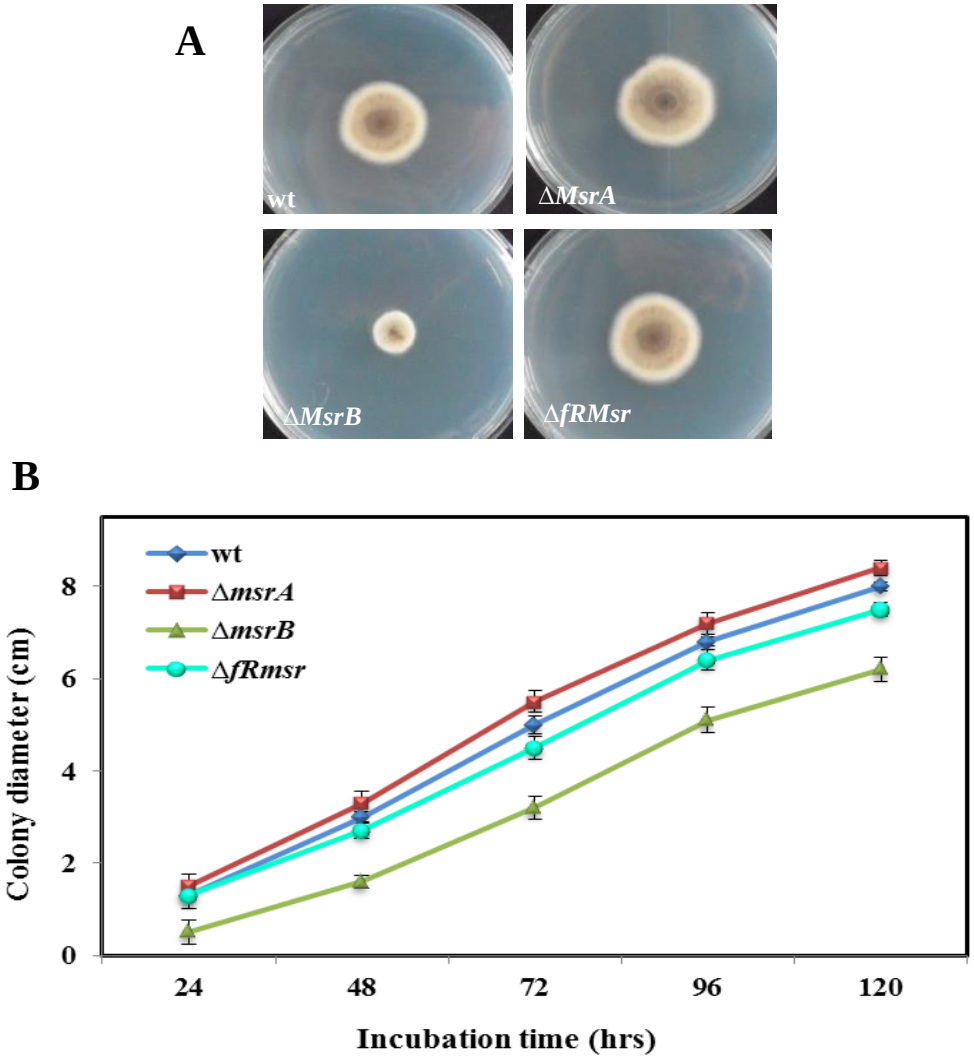

Fig. 12: Growth rate of *A. nidulans* Δ*msrA*, Δ*msrB* and Δ*fRmsr* knockouts in comparison with the wild type (TNO2a3). 10^4 conidiaspore were point inoculated on AMM agar plates and incubated at 37 °C for 3 days or 5 for Δ*msrB*. **(A)** The size of the colonies after 2 day of incubation. **(B)** Course of growth over 120 h (n = 3).

3.2.2 The biological significance of Msrs in *A. nidulans*

A major role of methionine sulfoxide reductase enzymes is to protect the cell against oxidative stress. To address this function, zone inhibition assays were performed (as given in Materials and Methods) to compare the sensitivity of the knockouts to H_2O_2, chloramine-T and menadione.

3.2.2.1 H_2O_2 and growth inhibition zone assay

Hydrogen peroxide (H_2O_2) is the simplest peroxide; it is also a strong oxidizer and it is considered a highly reactive oxygen species. The sensitivity to H_2O_2 increased with the concentration from 0.5 M to 4.0 M for all strains (wt, Δ*msrA*, Δ*msrB* and Δ*fRmsr*), which is reflected by the size of the inhibited area (Fig. 13 and Fig. 14). Among the msr knockouts strains Δ*msrB* was the most sensitive strain followed by Δ*msrA*, whereas Δ*fRmsr* was less affected compared with the wild type. In comparison of each concentration with the wild type; Δ*msrA* was most affected by 1.0 M, Δ*msrB* affected the most by treated with 2.0 M while Δ*fRmsr* was most affected by 1.0 and 2.0 M H_2O_2. The effects of H_2O_2 on MSRs knockouts Δ*msrA*, Δ*msrB* and Δ*fRmsr* compared with the wild type clearly confirmed the protection role of MSRs in the cell.

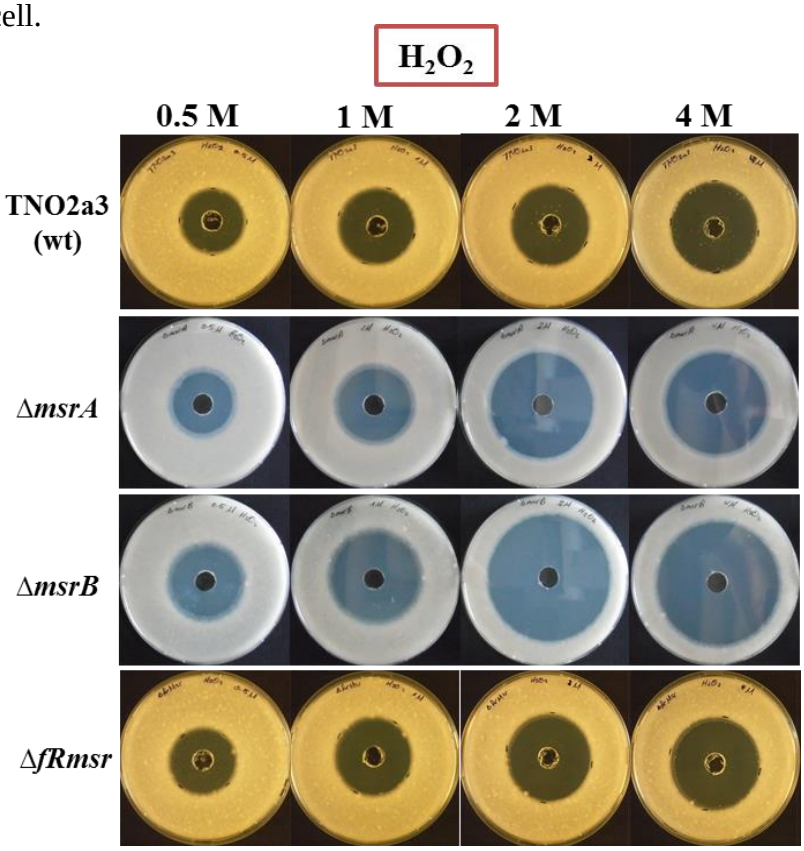

Fig. 13: Growth inhibition zone assay of Δ*msrA*, Δ*msrB* and Δ*fRmsr* compared with the wild-type strain TNO2a3. AMM agar plates treated with H_2O_2. $1x10^6$ spores were mixed with 48 °C-melted

AMM agar (15-20 ml) and a hole (diameter 1 cm) was created in the solidified agar. The size of the resulting inhibition zone was measured after 24 h of incubation at 37 °C.

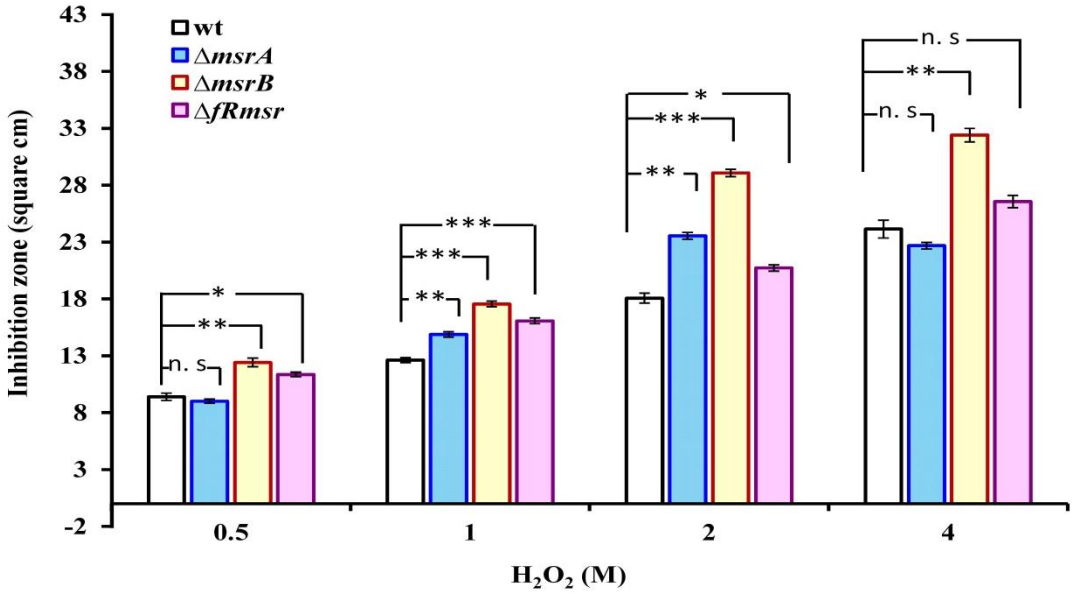

Fig. 14: Growth inhibition zone assay of Δ*msrA*, Δ*msrB* and Δ*fRmsr* compared with the wild-type strain TNO2a3. Growth inhibition by H_2O_2 was determined as in Fig. 13. The SEM was calculated from three experiments. n.s.: not significant (P >0.05); * <0.05; ** <0.01; *** <0.001.

3.2.2.2 Menadione growth inhibition zone assay

Menadione (vitamin K3) is a pro-oxidant that generates superoxide anion ($O_2^{-\cdot}$) through redox cycling (Liu et al., 1996). As such, menadione can be used as an inducer of oxidative stress (Lara-Rojas et al., 2011). The sensitivity to menadione was different with each concentration and for each strain and overall the effect of menadione was stronger than H_2O_2 on all strains (Fig. 15 and Fig. 16). The size of the inhibited area increased with the concentration; however, the difference between strains under investigation was small except for Δ*msrB*, which showed more sensitivity than all other strains. At the lowest conc. (10 mM), Δ*msrA* and Δ*fRmsr* showed sensitivity and Δ*msrB* was hardly able to grow compared with the wild type, whereas on the higher conc. (20, 30 and 60 mM) the growth of Δ*msrB* was completely inhibited. The treatment with menadione showed that Δ*msrB* is a hypersensitive strain compared with Δ*msrA*, Δ*fRmsr* and the wild type (TNO2a3). The effects of menadione on MSRs knockouts Δ*msrA*, Δ*msrB* and Δ*fRmsr* compared with the wild type reflected the significance of these enzymes for the conidia to counter act the stress. The data also showed that without MsrB the conidia would not survive under stress conditions.

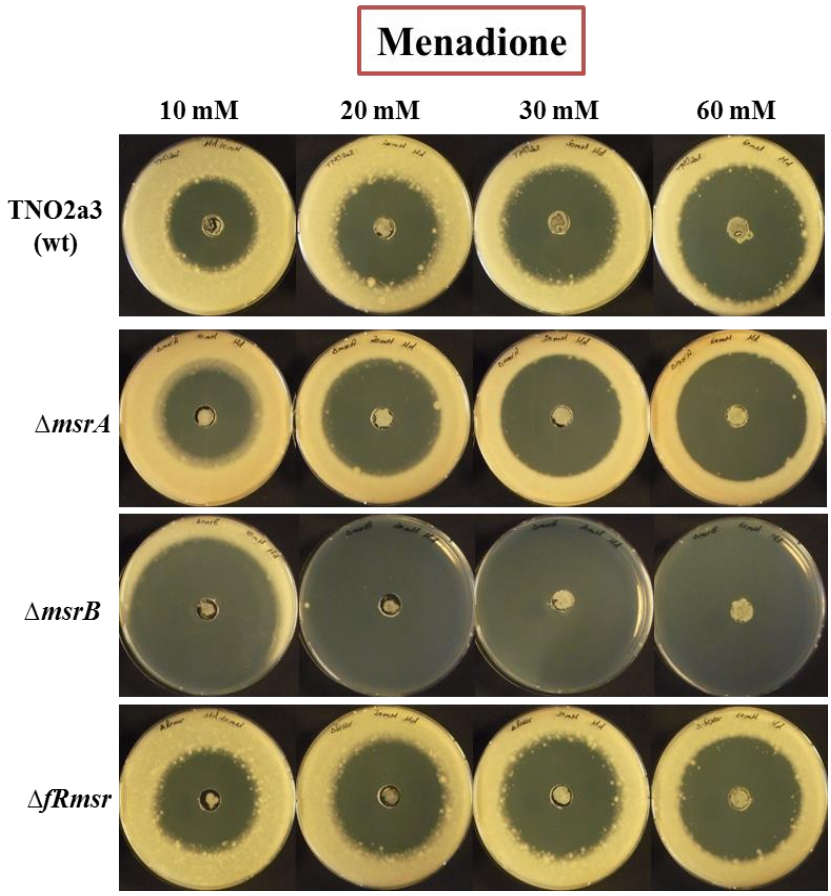

Fig. 15: Growth inhibition zone assay of Δ*msrA*, Δ*msrB* and Δ*fRmsr* compared with the wild-type strain TNO2a3. AMM agar plates treated with Menadione. $1x10^6$ spores were mixed with 48 °C-melted AMM agar (15-20 ml) and a hole (diameter 1 cm) was created in the solidified agar. The size of the resulting inhibition zone was measured after 24 h of incubation at 37 °C.

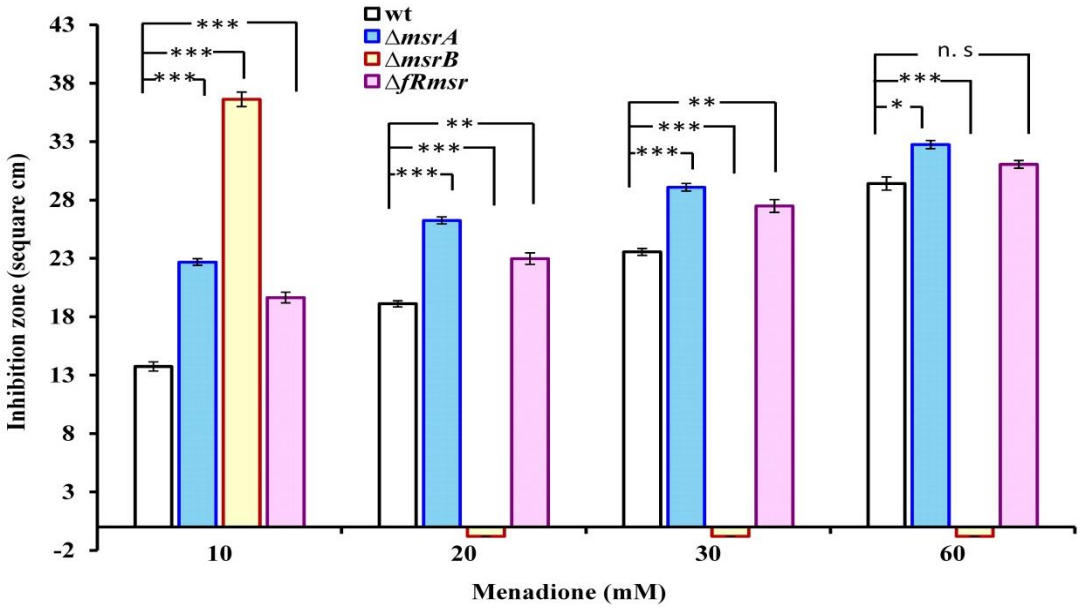

Fig. 16: Growth inhibition zone assay of Δ*msrA*, Δ*msrB* and Δ*fRmsr* compared with the wild-type strain TNO2a3. Growth inhibition by menadione was determined as in Fig. 15. The SEM was calculated from three experiments. n.s.: not significant (P >0.05); * <0.05; ** <0.01; *** <0.001.

3.2.2.3 Chloramine-T growth inhibition zone assay

Tosylchloramide or *N*-chloro tosylamide, sodium salt, sold as chloramine-T, is an N-chlorinated and N-deprotonated sulfonamide used as a biocide (fungicide; including spores) and a mild disinfectant. Chloramine-T (Ch-T) was used by the Heinemann group in many previous studies as a Met-preferring oxidant (Schlief et al., 1996; Ciorba et al., 1997; Su et al., 2007). The sensitivity to chloramine-T was concentration dependent and varied between all and tested strains (Fig. 17 and Fig. 18). The inhibition continuously increased with concentration for wt and Δ*fRmsr* strains while the effect of Ch-T on Δ*msrA* and Δ*msrB* was characterized by a very sudden increase of effect above 0.5 M (Δ*msrA*) and above 2 mM (Δ*msrB*), respectively. Interestingly, at the lowest concentration of Ch-T (0.5 M) Δ*fRmsr* was more sensitive than the wild type (t-test: P <0.01), but the increase of inhibition with higher concentrations was similar to the wild type

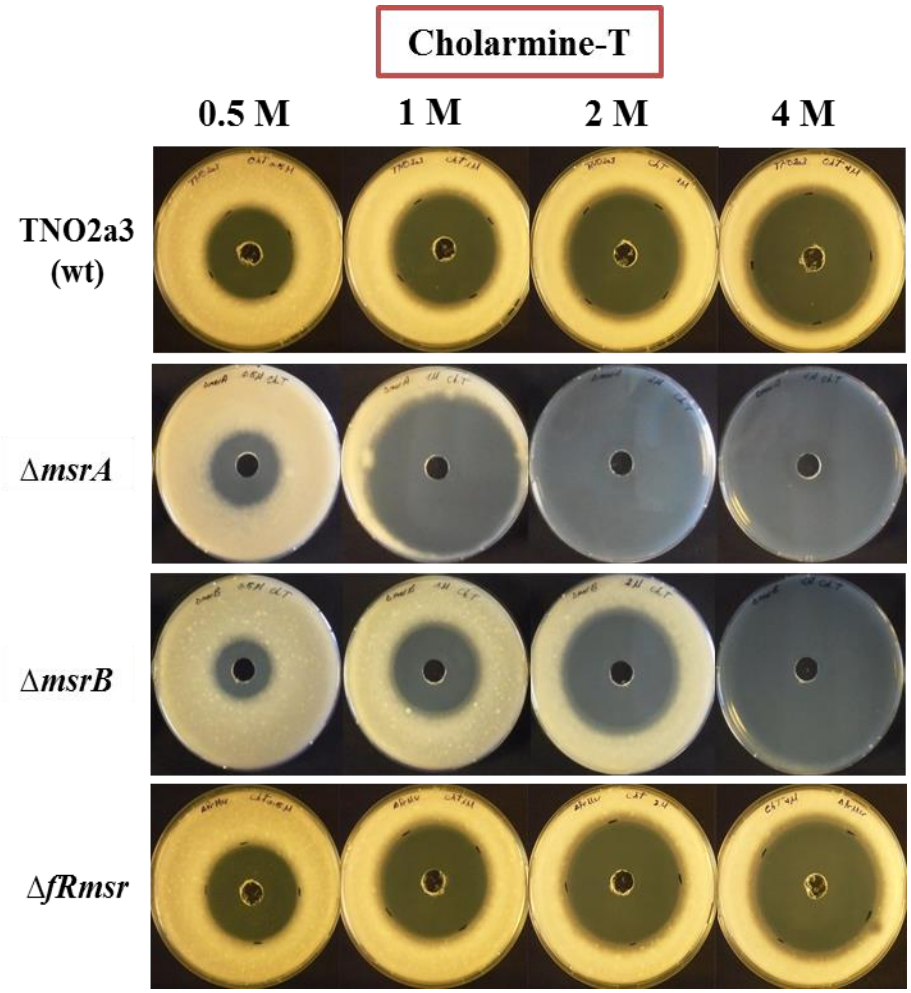

Fig. 17: Growth inhibition zone assay of Δ*msrA*, Δ*msrB* and Δ*fRmsr* compared with the wild-type strain TNO2a3. AMM agar plates treated with Ch-T. 1x10^6 spores were mixed with 48 °C-melted AMM agar (15-20 ml) and a hole (diameter 1 cm) was created in a solidified agar. The size of the resulted inhibition zone was measured after 24 h of incubation at 37 °C.

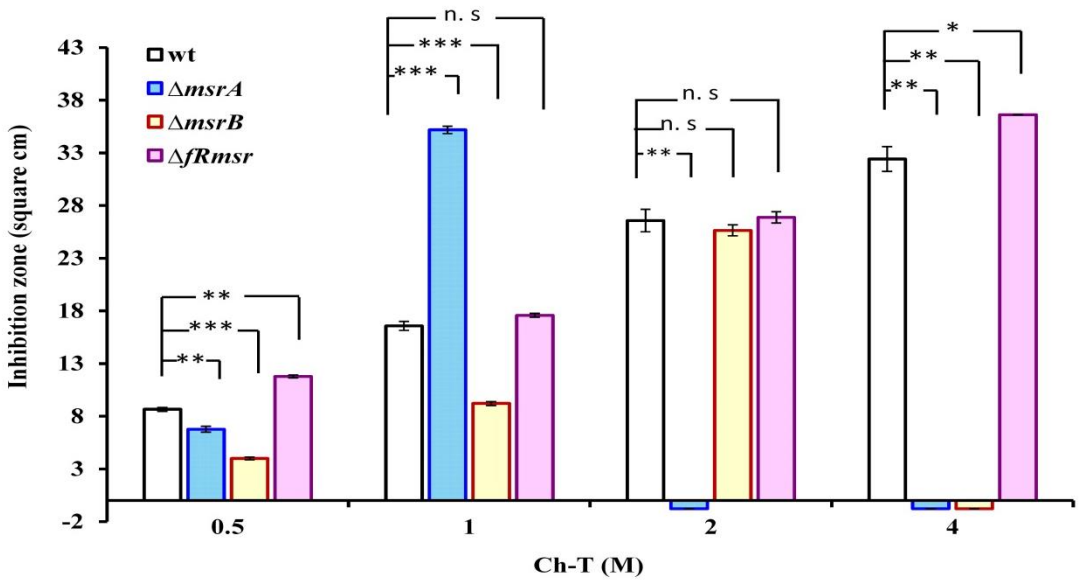

Fig. 18: Growth inhibition zone assay of Δ*msrA*, Δ*msrB* and Δ*fRmsr* compared with the wild-type strain TNO2a3. Growth inhibition by Chloramine-T was determined as in Fig. 17. The SEM was calculated from three experiments. n.s.: not significant (P >0.05); * <0.05; ** <0.01; *** <0.001.

3.2.3 *msrs* gene expression and oxidative stress

In order to investigate the possible regulation of *msr* gene expression by oxidative stress, mRNA of *msrA*, *msrB* and *fRmsr* genes were quantified in the wild-type strain TNO2a3. Using 1 x 10^7 conidispores, the wild-type strain was grown in AMM for 16 hours subsequently H_2O_2, chloramine-T or Paraquat was added to the culture and incubated at 37 °C for another 1 hour. As indicated in Figure 19, three different concentrations were used for H_2O_2 (25, 50, and 75 mM), and for chloramine-T (0.1, 1.0 and 10.0 mM), while two concentrations (5 and 10 mM) were used for paraquat. Total RNA was purified and cDNA was amplified as described in material and methods. The *A. nidulans* tubulin was used to normalize the expression level. Among all *msr* genes; *msrA* had the highest expression level followed by *fRmsr* and *msrB* under normal conditions (not shown).

The RT-PCR analysis showed that in response to oxidative stress, the level of all *msr* mRNA was dramatically increased. While expression of *msrA* was the highest at 0.5 mM H_2O_2 and gradually decreased along with increasing the concentration of H_2O_2 and Ch-T, the level of *msrA* mRNA was higher on the highest conc. (10 mM Ch-T) than in normal conditions. While the data showed that the mRNA level of *msrB* was the lowest in normal conditions; it showed that oxidative stress upregulated *msrB* expression especially by Ch-T. *fRmsr* expression was 10-fold more after treating with 25 mM H_2O_2 than on normal conditions without any

significant difference of using higher concentrations. Paraquat showed more effect on the expression of *fRmsr* than on *msrA* or *msrB.*

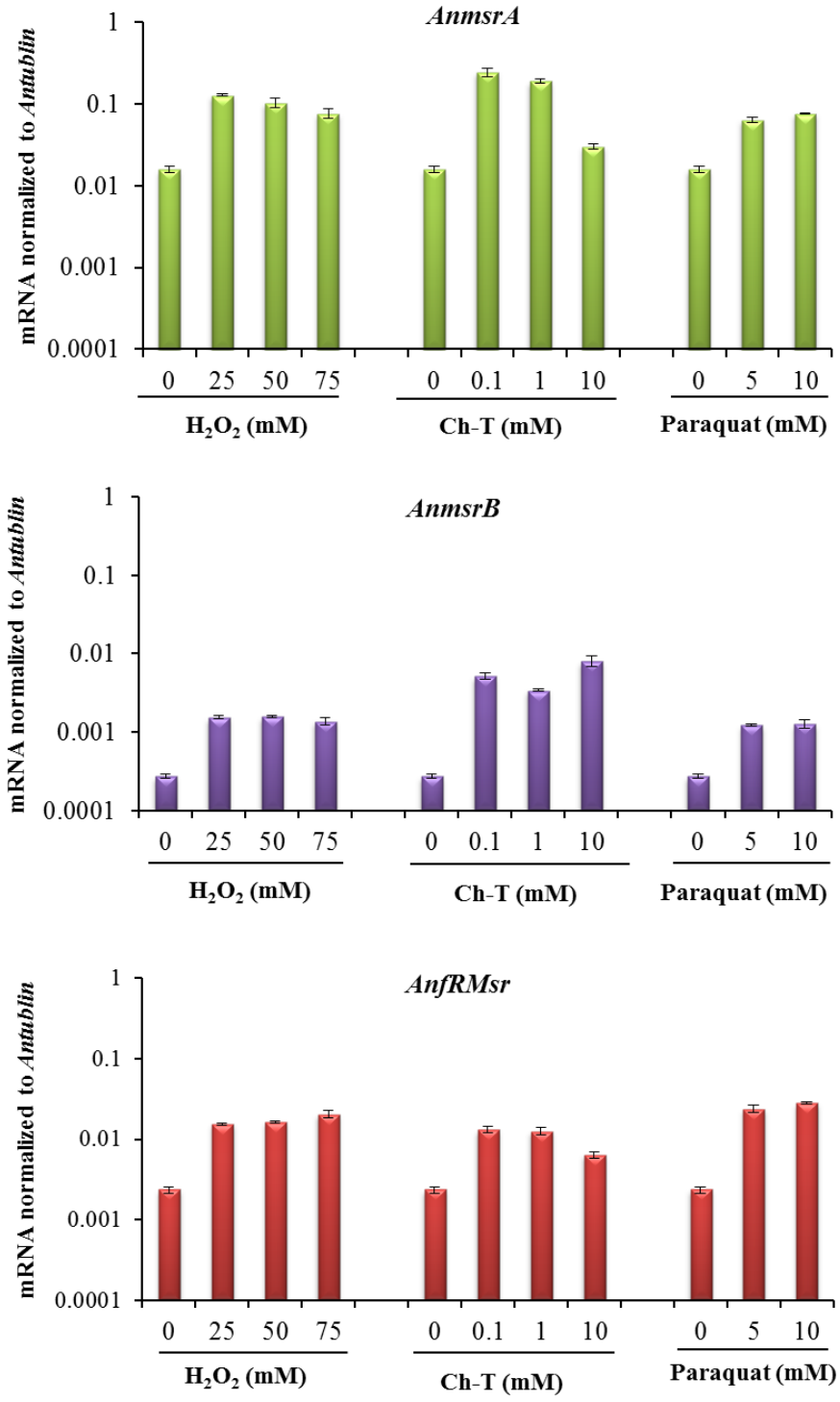

Fig. 19: Quantification RT PCR of *msrA, msrB* and *fRmsr* gene expression in *A. nidulans* TNO2a3 strain. The wild-type strain (TNO2a3) was grown in AMM for 16 hours then H_2O_2, chloramine-T or paraquat were added to the culture and incubated at 37 °C for another 1 hour.

3.3 Cloning and expression of *A. nidulans* Msrs

In order to characterize the *A. nidulans* MSRs enzymes, specific primers were used to amplify the full-length open reading frame of *msrA*, *msrB* and *fRMsr* from cDNA derived from strain TNO2a3. The DNA sequence of all final clones was confirmed by Sanger sequencing. For the expression of these enzymes in *E. coli*, the DNA fragments were cloned into the expression vector pET-15b and MsrA, MsrB and fRMsr proteins were successfully expressed in *E. coli* as recombinant His-tagged protein (Fig. 20).

Human Msr proteins were used for comparison in functional studies. Purification of the His-tagged human enzymes MsrA, MsrB2, and MsrB3 have been established and described previously (Kuschel et al., 1999; Jung et al., 2002; Hansel et. al., 2003). Expression of the cloned fungal genes was successful in the BL21-CodonPlus strain, containing gene copies encoding tRNAs that frequently limit translation of heterologous proteins in *E. coli*. While expression of AnMsrB (18 kDa) and AnfRMsr (23 kDa) in this strain resulted in stable and soluble proteins, full-length AnMsrA (26 kDa) could not be isolated as stable protein, the product appeared with two bands in SDS-PAGE. Hansel et al. (2002) previously identified a mitochondria-targeting sequence at the N-terminus of human MsrA, which was found to limit the solubility upon expression in *E. coli*. Along with that the protein yield was very less. Accordingly, we deleted the first 18 amino acids of AnMsrA and the truncated protein (24 kDa); starting from the second Met in the reading frame could be expressed in stable and soluble form.

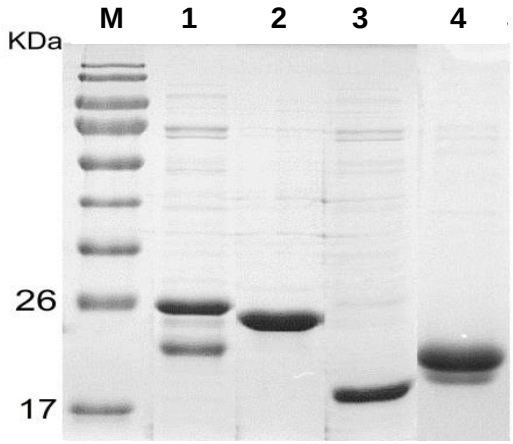

Fig. 20: Recombinantly expressed MSRs from *A. nidulans*. Purification of recombinant MsrA, MsrB and fRMsr proteins with His-Tag in *E. coli;* lane M: Fermentas protein ladder, 1: MsrA, 2: truncated version of MsrA, 3: MsrB, 4: fRMsr.

3.3.1 Enzymatic activity of *A. nidulans* Msrs

The activity of the recombinant AnMsrA, AnMsrB and AnfRMsr proteins was tested using a synthetic peptide containing oxidized methionine (Ac-K-I-F-M(O)-K-Dnp) with average mass of 933 Da. The peptide carried an acetyl group at the N-terminus, whereas the C-terminus end was linked to dinitrophenol (DNP). This chromophor was used for peptide monitoring at 365 nm wavelength. The peptide preparation is a mixture, representing the S and R isoforms of methionine sulfoxide in approximately 50% ratio. Chao et al., (1997) demonstrated a linear correlation between protein hydrophobicity and the conversion of Met residues to MetO, thus reversed phase chromatography can be used to separate the Met-containing peptides based on the oxidation state, without separation of the S/R epimers (Haenold et al., 2007).

Following incubation of the oxidized peptide with the respective enzyme preparation, reduced and oxidized peptides were separated by reversed-phase chromatography and the ratio of the reduced and oxidized elution peak was determined as readout of the enzymatic activity (Fig. 21). Both peaks were clearly separated in the acetonitrile gradient, with the reduced and more hydrophobic peptide being eluted later than the oxidized peptide. Quantitative analysis of the peaks showed that 50% of the oxidized peptide was reduced after incubation with only AnMsrA or AnMsrB (Fig. 21B and 21C). As mentioned above, there were two products of AnMsrA, a long one (a product of the full length) and the truncated one, we found that both of them were active and were able reduce MetO in peptide. However, all the experiments of this study were performed using the truncated version. By contrast, AnfRMsr was unable to reduce the MetO-containing peptide; the single peptide peak was unchanged upon incubation with the enzyme (Fig. 21D). This confirms the ability of *A. nidulans* MsrA and MsrB proteins to reduce methionine sulfoxide in peptide while no such activity was detectable for fRMsr. Complete reduction of the oxidized substrate peptide was achieved upon incubation with a combination of AnMsrA and AnMsrB to reduce both sulfoxide epimers (Fig. 21E). Similar results were obtained using human MsrA and MsrB3 in combination (Fig. 21F).

3.3.2 Specificity of *A. nidulans* Msrs

Separation of reduced and oxidized Met by reverse phase chromotagraphy relied on the coupled DNP group, excluding assays with AnfRMsr, as this enzyme acts only on free methionine. A different method needed to be used for confirming the enzymatic activity and the sterospecificity of AnfRMsr. Uthus (2010) developed a capillary electrophoresis (CE) assay for Msr enzymes based on the separation of the dabsyl-Met-O diastereomers as

substrates. Capillary electrophoresis is an analytical technique that separates ions based on their electrophoretic mobility with the use of an applied voltage.

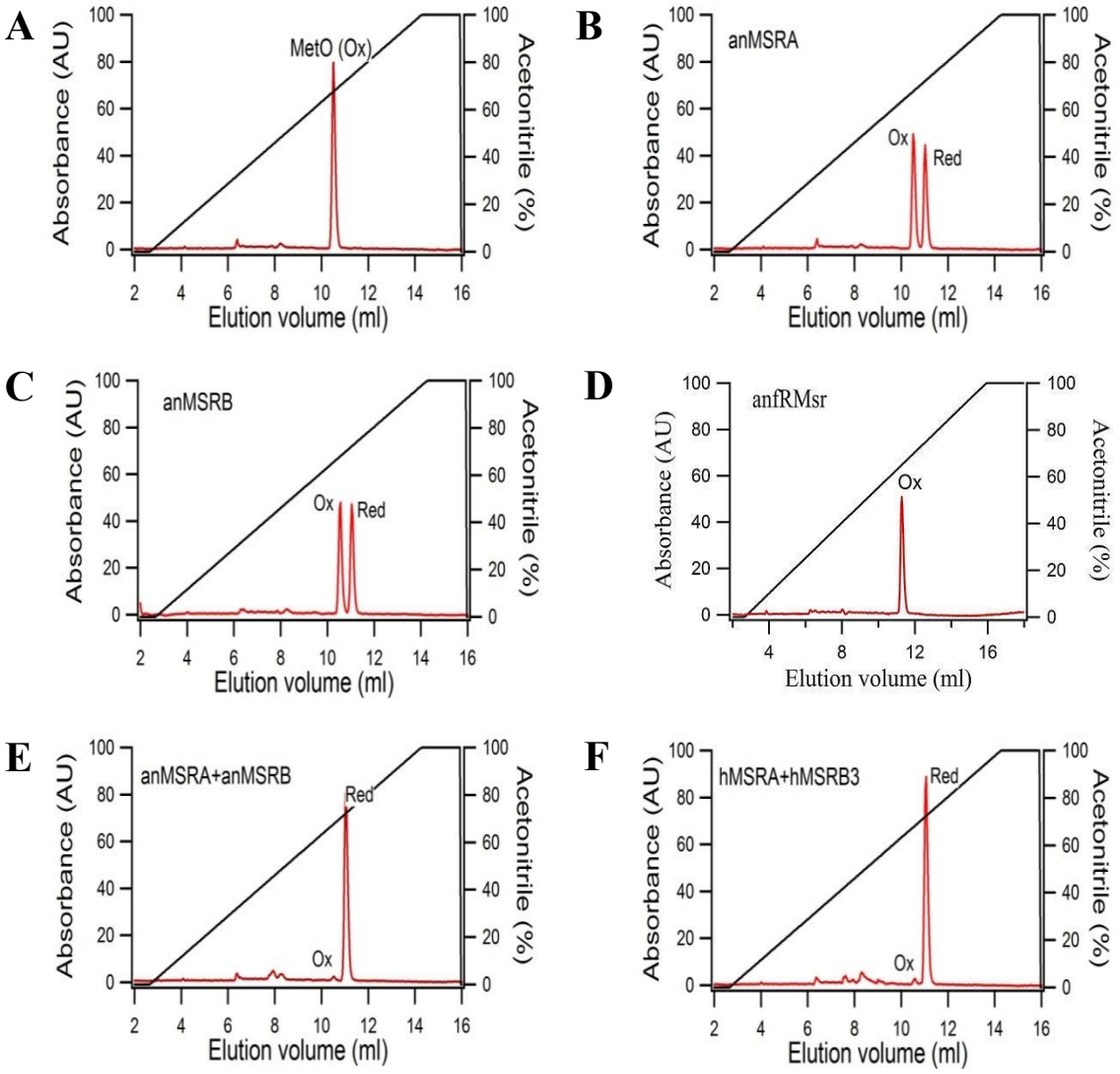

Fig. 21: Enzymatic activity of recombinantly expressed MSRs from *A. nidulans*. (A) Reverse-Phase HPLC chromatogram of the synthetic peptide (KIFM(O)k-Dnp) as detected at 365nm. **(B-F)** RP HPLC chromatogram of the synthetic peptide (MetO) oxidized form of Methionine after the incubation with the recombinant proteins for 30 min at 37 °C in the presence of 20 mM DTT and 50 mM Tris pH 8,0. **(B)** AnMsrA, **(C)** AnMsrB, **(D)** AnfRMsr, **(E)** AnMsrA and AnMsrB, and **(F)** hMsrA and hMsrB3. Ox: oxidized peptide; Red: reduced peptide.

The electrophoretic mobility is dependent upon the charge of the molecule, the viscosity, and the atom's radius (Righetti et al., 2013). The separation of the peptides using CE is perhaps the simplest and trouble-free aspect of capillary electrokinetic separations, and it also has a distinct advantage over RP-HPLC in that the latter usually fails in the separation of small similar polar peptides (Righetti et al., 2013). The method which was used by Uthus (2010)

also allowed the analysis of dabsyl-Met, the product of dabsyl-MetO reduction by Msr. In cooperation with Qingfu Zhu and Prof. Gerhard K. E. Scriba (Department of Pharmaceutical Chemistry, FSU Jena) a stable CE assay was developed to determine the activity of Msr using free Met-O as a substrate. In contrast to Uthus's method (2010), our developed method had the advantage of using free Met-O as a substrate, and in contrast to RP-HPLC this method allowed separation of diastereomers of Met-O. Substrate stereospecificity of the AnMsrA, AnMsrB and AnfRMsr enzymes was determined using a mixture of free Met-R-O and free Met-S-O.

The enzymatic reactions were performed at 37 °C for 15 min with the free MetO mixture in the presence of DTT followed by derivatization with dabsyl chloride and analyzed by capillary electrophoresis as described in Zhu et al. (2013). The electropherograms show that S- and R-isomers of free MetO were successfully separated and it confirmed the sterospecificity of each enzyme in comparison with a control sample (in the absence of the enzyme). The reduced Met peak only appears in the electropherogram when the enzyme was added to the reaction. The separated isomers resulted in two equal peaks, each representing one isomer, 90 % of the oxidized isomer peak was converted into the reduced Met peak after incubation with AnMsrA or AnfRMsr. By contrast, AnMsrB was able to convert only 10 % of the Met-R-O peak into the Met. This confirms the ability of *A. nidulans* MsrA to reduce the S-isomer of free methionine sulfoxide. This finding suggests that the main role of AnfRMsr is the reduction of R-isomer of free MetO, while AnMsrB is responsible for the reduction of R-isomer in peptide (Fig. 22).

3.3.3 pH-dependence of *A. nidulans* Msrs

The optimum pH was determined for AnMsrA, AnMsrB and AnfRMsr as well as for hMsrA, hMsrB2 and hMsrB3. The reactions for MsrAs and MsrBs were carried out at 37 °C with Ac-KIFM(O)K-Dnp peptide and DTT as a reductant and the result was monitored and analyzed by RP HPLC. For AnfRMsr, the reactions were performed using free Met-O as a substrate and DTT as a reductant. After the incubation for 30 min at 37 °C, reactions were derivatized with dabsyl chloride then analyzed using CE. The reactions were performed in the pH range from 5.0 to 10.0. The data revealed that the optimum pH is 8.0 at which both fungal and human Msr showed the highest activity (Fig. 23). All tested enzymes were almost inactive at pH values of 6.0 or below. While AnMsrA and AnMsrB showed 60 % activity at pH 7.5 and a slight decrease at pH higher than 8.0, AnfRMsr showed only 30 % at pH 7.5, and more pronounced decrease at pH higher than 8.0.

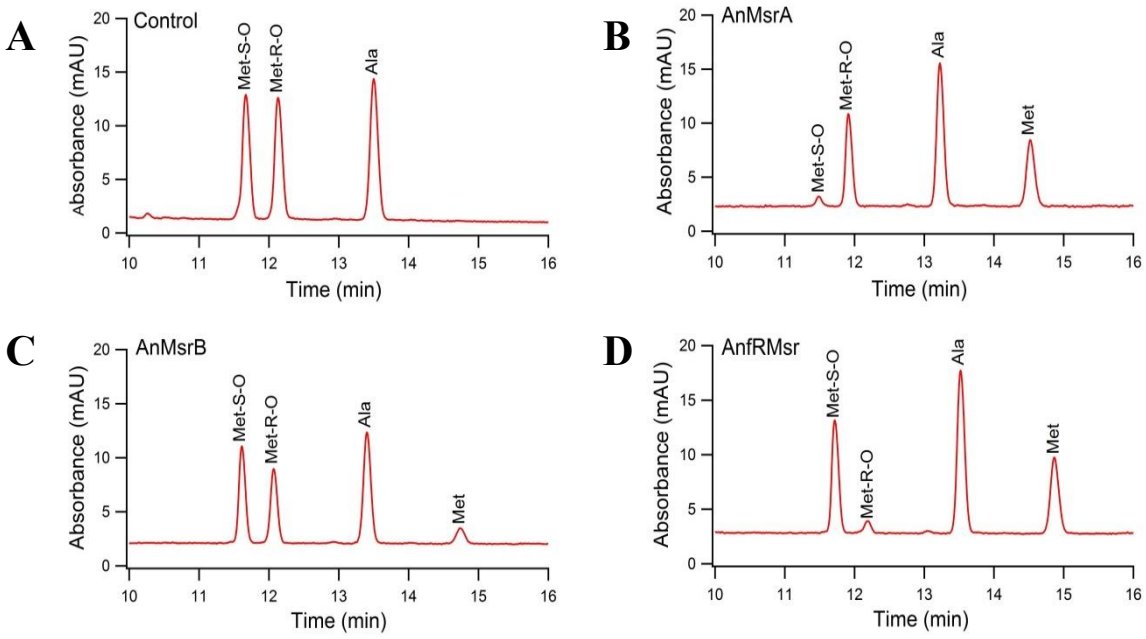

Fig. 22: Enzymatic activity of recombinantly expressed Msrs from *A. nidulans* using capillary electrophoresis. (A) Electropherogram of free MetO derivatized with dabsyl chloride and β-Ala (Ala) as internal standard. **(B-D)** Electropherogram of free MetO after incubation with AnMSR for 30 min at 37 °C in 50 mM Tris/HCl (pH 8) and 20 mM DTT as enzyme reductant then derivatized with dabsyl chloride and β-Ala as internal standard.

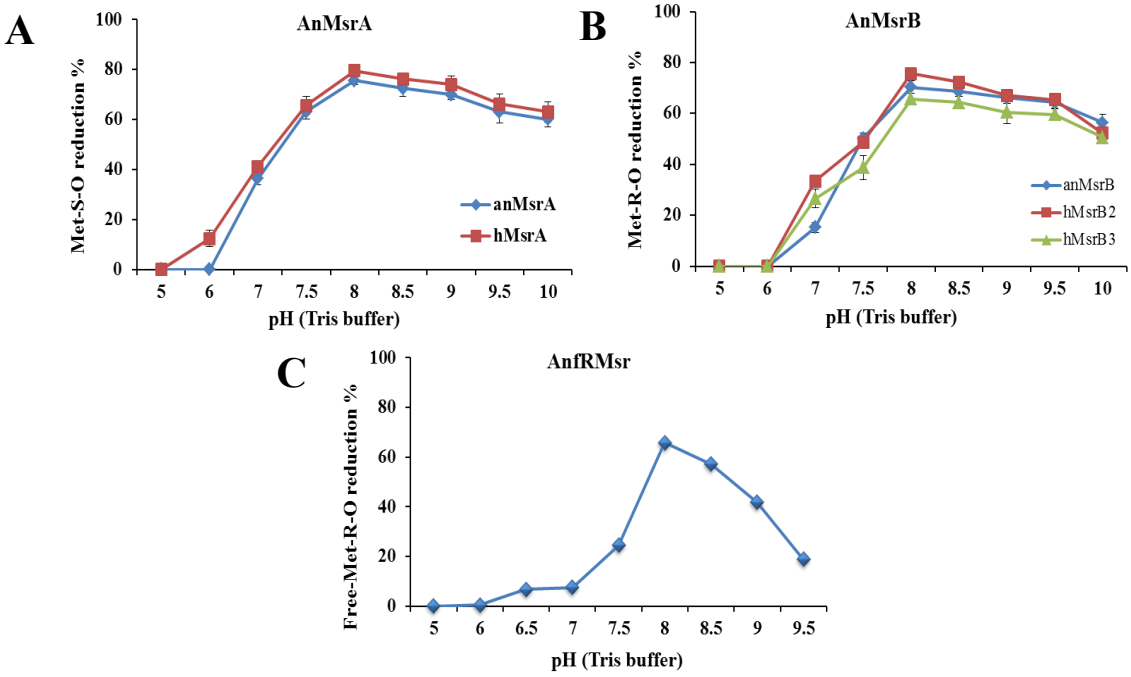

Fig. 23: pH-dependence of *A. nidulans* Msr. Activity of human and fungal MsrA **(A)** was determined at 37 °C for 30 min using 3 nmol peptide (KIFM(O)K-Dnp) in the presence of 20 mM DTT and 0.1 M Tris/HCl adjusted to the indicated pH ratio. The reaction was then analyzed by RP HPLC at 365 nm. **(B)** The same analysis as MsrA carried out for fungal MsrB and human MsrB2 and MsrB3. **(C)** fRMsr, reaction was carried out in 0.1 M Tris/HCl at the indicated pH levels and 1 M free Met-O in the presence of 20 mM DTT at 37 °C for 30 min and then derivatized by dabsyl chloride. The reactions were then analyzed by CE.

3.3.4 Enzymatic activity of recombinantly expressed fRMsr

In the assays described before (RP-HPLC and CE), enzymatic activity of Msr enzymes was determined with DTT as reducing agent in the reaction mixture. Under physiological conditions, however, recycling of the oxidized Msr enzymes depends on thioredoxin (Trx) and thioredoxin reductase (TrxR) as depicted in Fig. 24A. Fig. 24B shows the enzymatic activity of AnfRMsr with free Met-O in a coupled assay with recombinant TrxA and TrxR from *A. nidulans* and NADPH as reducing substrate. In this assay, the reaction is monitored via the loss of absorption at 340 nm due to conversion of NADPH to $NADP^+$. The reactions with variable substrate concentrations were prepared at room temperature in a phosphate buffer at pH 8.0. Fig. 24 C shows the determined reaction velocities as function of the substrate concentration. The Km value, was calculated according to the Michaels-Menten equation, was 0.63 ± 0.3 mM.

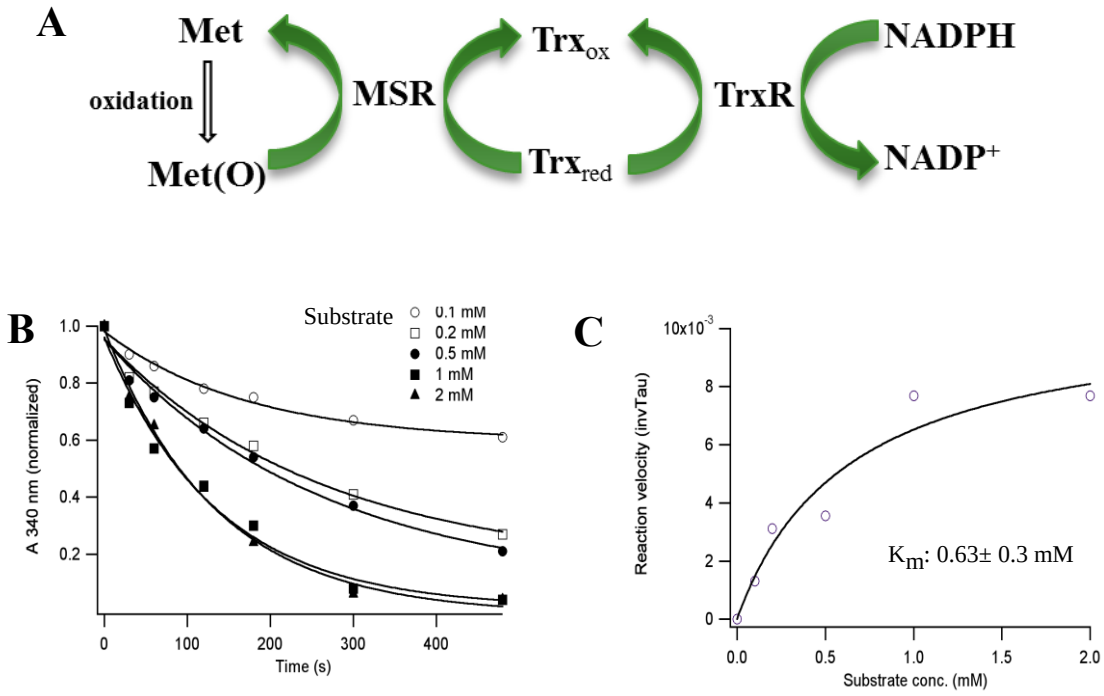

Fig. 24: Enzymatic activity and biochemical properties of AnfRMsr. (A) A diagram presenting the reduction of Msr by the Trx/TrxR system. **(B)** Activity of AnfRMsr was measured using the coupled assay with different free MetO concentration. **(C)** Substrate saturation curve. Km value was determined by fitting experimental data to the Michaelis-Menten equation.

3.3.5 Identification of catalytic cysteine of AnMsr

In order to investigate the Cys residues involved in the *A. nidulans* Msr catalysis, the conserved Cys of each enzyme were separately mutated to Ser. The mutated enzymes were cloned and expressed as previously described for the wild-type enzymes, followed by protein

purification. Wild-type and mutated Msr proteins were pure as evaluated by SDS-PAGE, and their ability to reduce the Met-O containing peptide was determined using RP-HPLC or using the coupled assay for AnfRMsr.

3.3.5.1 Catalytic cysteine of AnMsrA

The alignment data showed that AnMsrA has only four Cys (Fig. 6). Site directed mutagenesis was used to substitute all of them individually to Ser. As shown on the diagram, two of them are highly conserved among all MsrAs (Fig. 25). One of these two is Cys52 located in the MsrA signature sequence (GCFWG); this residue was previously identified as the catalytic Cys for MsrA (Weissbach et al., 2002; Hansel et al., 2002). The second widely conserved Cys is Cys91 without any known function for any MsrA structure known, also for *A. nidulans* replacing it with Ser it showed no effect on the activity of AnMsrA. The effects of replacing the Cys with Ser and the ability of mutated enzymes for using either of DTT or Trx/TrxR system as a reductant were investigated. The data revealed that Cys52 is the only critical Cys for the catalytic activity of AnMsrA using either Trx/TrxR system or DTT as a reductant (Table 1). While the data indicated that Cys52 and Cys200 inactivate the AnMsrA using Trx/TrxR system as a reductant, it also showed that Cys200 had no effect on the activity when DTT used as a reductant. Also substitution of Cys78 by Ser had no effect on the activity. Our data suggest that Cys52, which is located at the N-terminus, is the catalytic Cys (CysA), whereas Cys200 at the C-terminus is the recovery Cys (CysB). It also suggests that while DTT does not require a disulfide bridge to recover the sulfenic acid of AnMsrA and it can directly react with CysA, while Trx requires the disulfide bridge to recover AnMsrA.

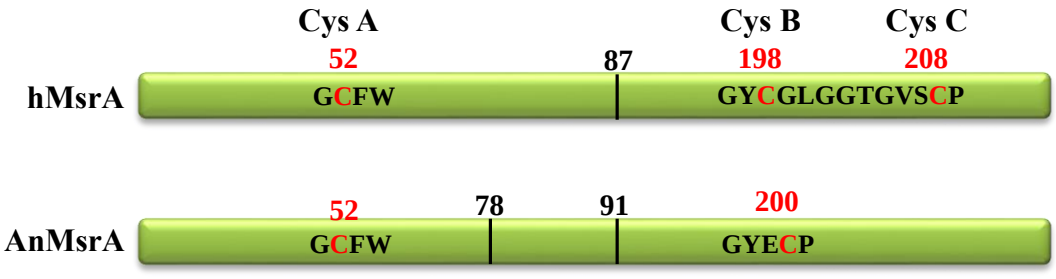

Fig. 25: Diagram showing the Cys position in AnMsrA in comparison with hMsrA. All the Cys are shown on the diagram; the catalytic Cys52 and recycling Cys200 are shown for AnMsrA. The colors of the diagram are as follows; red color represents the functional or the conserved Cys and black color represents the non-conserved Cys.

Table 1: Site directed mutagenesis for the Cys residues in the active site of AnMsrA. The activity was examined using KIFM(O)K-Dnp peptide and the results was analyzed by using RP-HPLC. (+++) represents the ability of AnMsrA to reduce more than 80% of Met-S-O in 30 min at 37 °C, while (---) represents a less than 10% reduction of Met-S-O.

	DTT	Trx/TrxR
AnMsrA (wt)	+++	+++
AnMsrA_C52S	---	---
AnMsrA_C87S	+++	+++
AnMsrA_C91S	+++	+++
AnMsrA_C200S	+++	---

3.3.5.2 Catalytic cysteine of AnMsrB

In contrast to human MsrA (hMsrA), hMsrB2 and hMsrB3 require one Cys for the catalysis of Met-R-O. This Cys123 (*A. nidulans* MsrB) is highly conserved among the MsrBs, and it is the catalytic Cys for all known MsrB (Kim and Gladyshev 2005). The alignment data revealed that the number of Cys is varied among MsrBs (Fig. 7), however the conserved Cys are only five including the catalytic Cys and a Zn-binding motif (2 pair of CxxC). Site-directed mutagenesis was used to mutate the highly conserved Cys to Ser (Fig. 26), and the activity of the mutated enzyme and its ability to either use DTT or Trx/TrxR system as a reductant were investigated. The data showed that the presence of Ser instead of Cys123 inactivate the AnMsrB using DTT as a reductant as well as Trx/TrxR system (Table 2). The data suggest that Cys123 is crucial for the activity and it is the catalytic Cys of AnMsrB. A study on MsrBs from Drosophila and mammals stated that MsrB is a zinc-dependent enzyme, and it has a Zn-binding site organized in two CxxC motifs (xx could be any amino acids) (Kryukov et al., 2002). The alignment showed that the Zn-binding motif (C50xxC53 and C98xxC101 in AnMsrB) are highly conserved in all MsrBs. To further investigate the mutation effect at this motif we mutated one Cys of each motif (Cys53 and Cys101) into Ser. The results indicated that mutating any Cys at Zn-binding sites resulted in complete loss of AnMsrB activity in DTT- or Trx-dependent reaction.

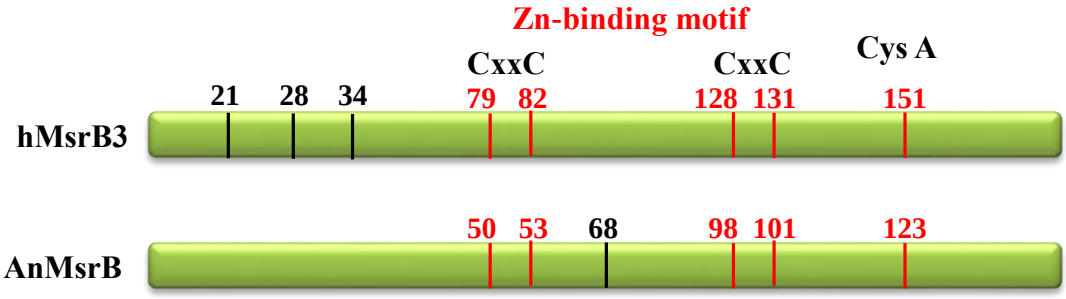

Fig. 26: Diagram showing the Cys position of AnMsrB in comparison with hMsrB3. All the Cys are shown on the diagram; the catalytic Cys123 and Zn-binding motif (2 pairs of CxxC) are shown for AnMsrB. The colors of the diagram are as follows; red color represents the functional or the conserved Cys and black color represents the non-conserved Cys.

Table 2: site directed mutagenesis for the Cys residues in the active site of AnMsrB. The activity was examined using KIFM(O)K-Dnp peptide and the results was analyzed by using RP-HPLC. (+++) represents the ability of AnMsrB to reduce more than 80% of Met-R-O in 30 min at 37 °C, while (---) represents a less than 10% reduction of Met-R-O.

	DTT	Trx/TrxR
AnMsrB (wt)	+++	+++
AnMsrB_C123S	---	---
AnMsrB_C53S	---	---
AnMsrB_C101S	---	---

3.3.5.3 Catalytic cysteine of AnfRMsr

The alignment shows that the number of Cys varied among free R-Methionine sulfoxide reductases (Fig. 8). However, fungal fRMsr shares all the Cys with yeast fRMsr except the latter has one extra Cys in the N-terminus (Fig. 27A). Previous studies showed that only three Cys are functional for yeast fRMsr, and these 3 Cys (Cys91, Cys101 and Cys125) were found to underlie the catalytic mechanism of yeast fRMsr; (Le et al., 2009; Kwek et al., 2010). We mutated the three Cys of AnfRMsr Cys104, Cys114 and Cys138 individually to Ser. The ability of the mutated enzymes to reduce free MetO was examined the Trx/TrxR coupled assay as mentioned above in section 3.3.4. The data showed that substitution of Cys104 and Cys138 by Ser resulted in complete loss of AnfRMsr activity using Trx/TrxR system as a reductant (Fig. 27B). In contrast to yeast fRMsr, the results showed that substitution of Cys114 to Ser has no effect on the enzyme activity suggesting that only Cys104 and Cys138 are involved in the catalytic mechanism of AnfRMsr.

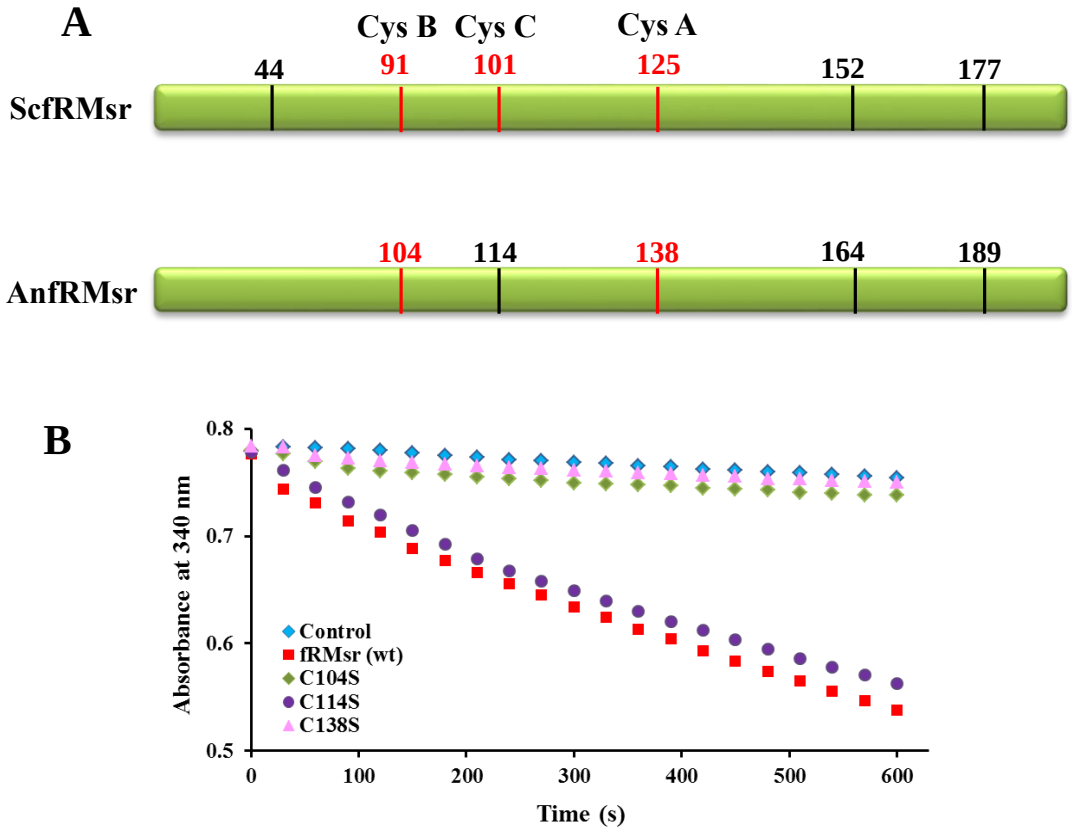

Fig. 27: *A. nidulans* fRMsr functional Cys. (A) Diagram showing the Cys position of AnfRMsr in comparison with ScfRMsr. All the Cys are shown on the diagram. The color of the diagram are as follows; red color representing the functional Cys and black color representing the non-functional Cys. **(B)** NADPH-dependent activity of Cys-mutated AnfRMsr in comparison with wild type enzyme was followed by the decrease in NADPH absorbance at 340 nm. Control sample was measured with all reaction mixture except for the AnfRMsr.

3.4 Msr and peptide specificity

It is widely known that MsrA and MsrB enzymes differ in their specificity to peptide substrates versus free methionine sulfoxide as a substrate. However, the question if the sequence context surrounding the oxidized Met in a peptide affects the enzymatic activities of Msrs is largely unknown. For MsrA, Sun et al., (1999) reported that this enzyme preferentially binds and repairs methionine sulfoxide with hydrophobic sequences. They compared the reduction rate for all methionine residues in calmodulin and found a strong correlation between hydrophobicity and repair by MsrA. To further address the relevance of hydrophobicity, we used the peptide with the best reduction rates in the Sun et al., study (GTVMRSL) and designed the replaced of arginine by valine to increase hydrophobicity or by aspartate to reverse the charge. All three peptides were synthesized with a lysine-flanking residues and a C-terminal DNP-label. As shown in Fig. 28, in a reduction assay with human MsrA, the more hydrophobic peptide (KGTVM(O)VSLK) was indeed reduced faster than the

original R-containing peptide. Interestingly, despite a similar contribution to hydrophobicity, aspartate in the peptide clearly reduced the reduction rate in comparison to arginine. Thus hydrophobicity might not be the only relevant factor affecting the substrate preference of MsrA.

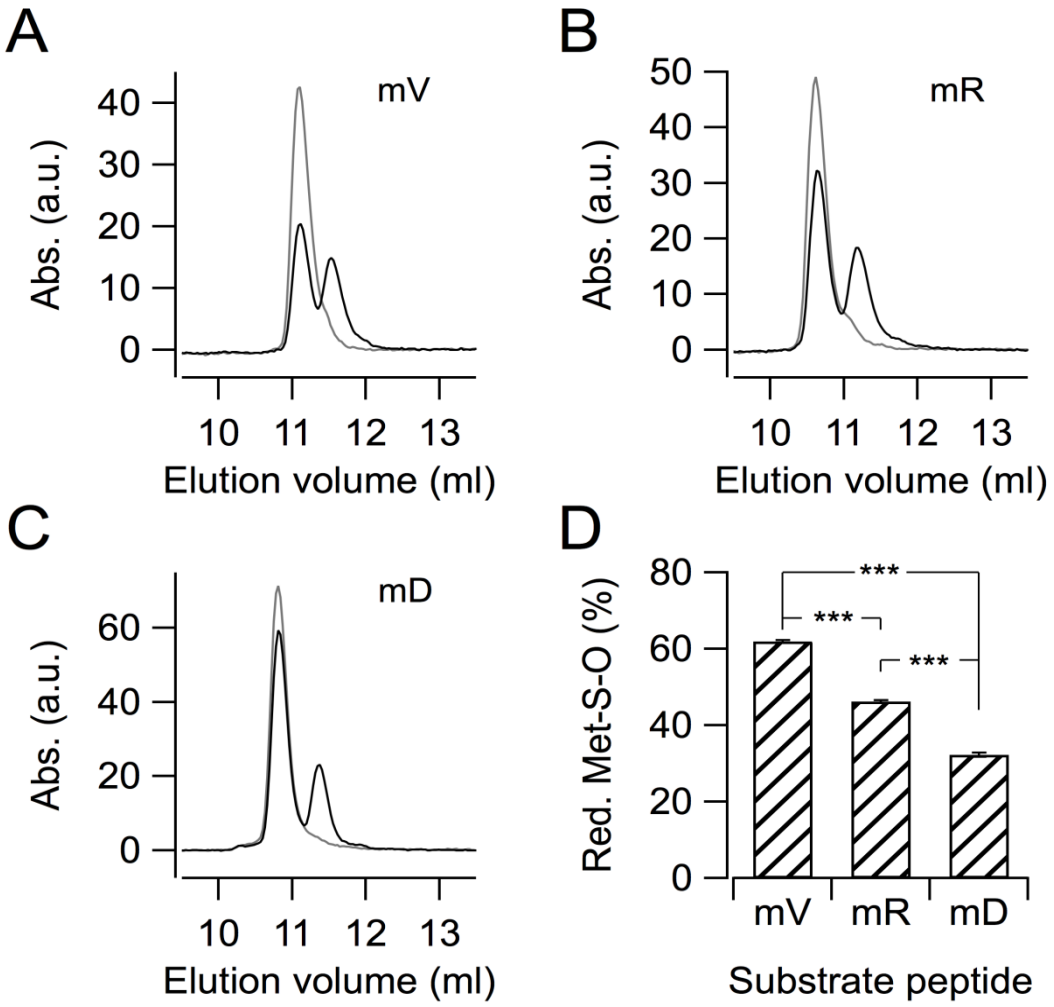

Fig. 28: Influence of Arg, Val, or Asp at position +1 of Met on the MetO reduction by hMsrA. Synthetic peptides containing oxidized methionine (mixture of R- and S-isomers) were subjected to reduction by hMsrA. The reactions were performed with 2 µg of the recombinant protein for 15 min at 37 °C in the presence of 20 mM DTT and 50 mM Tris (pH 8). **(A-C)** RP-HPLC chromatograms of the peptides before (grey peak) and after (black peaks) the incubation with hMsrA. mV: KGTVM(O)VSLK, mR: KGTVM(O)RSLK and mD: KGTVM(O)DSLK. **(D)** The calculated reduction percentage. The SEM was determined from three independent measurements.

To further investigate the impact of neighboring amino acid of MetO, we designed a set of peptides with a different residue at position -1 and/or +1. As stated in the material and methods (Section 2.5.1), the peptides were designed to investigate specific questions. These questions included the impact of the presence of acidic, basic, hydrophobic or aromatic amino acids next to MetO on the activity of MsrA and MsrB from human and *A. nidulans*.

To ensure solubility and to favor a stretched-out structure, lysine residues were incorporated at position -2 and +2 of most peptides. All peptides were labeled with DNP at the carboxyl-terminus, to allow photometric detection after RP-chromatography. This peptide design had been introduced previously, using the peptide sequence KIFM(O)K-(DNP) (Haenold et al. 2007). As shown in Fig. (29A and 29B), a peptide with two polar, uncharged residues (N) flanking the oxidized methionine was equally-well reduced as this original KIFM(O)K peptide. Comparison of the peptides KNM(O)NK and NM(O)N showed that the flanking lysines have only minor influence on the substrate acceptance. Also comparison of the peptides NM(O)N and VDM(O)D showed that presence of two-acidic residues flanking the MetO significantly reduced the ability of MsrAs to repair MetO. The reduction of the shorter NM(O)N peptide by human MsrA was only slightly reduced and the fungal MsrA did not significantly differentiate both substrates.

3.4.1 Peptide specificity profile of MsrA enzymes

The ability of hMsrA and AnMsrA to reduce Met-S-O in peptides in the context of different flanking amino acids was examined. As shown in Fig. (31), both enzymes showed very similar patterns of selectivity ($P = 0.998$). The results clearly showed that both hMsrA and AnMsrA are able to discriminate between different peptides.

Interestingly, the reaction rates with both MsrA enzymes were diminished when both polar asparagine residues were replaced by the hydrophobic residues phenylalanine or valine. By contrast, introduction of additional positive charges (K) in the -1 and +1 positions further accelerated the substrate reduction by both types of MsrA. Thus, for the direct neighbors of the substrate Met(O), hydrophobicity is no promoting factor, but decreases the substrate turnover rate. We then asked if charged residues per se promote the peptide-enzyme interaction or if the type of charge is decisive. Introduction of negatively charged glutamic acid residues on both sides of Met(O) dramatically reduced the substrate quality, the turnover in typical 15 min assays was even below the VM(O)V peptide. However, a single glutamate on either side, combined with lysine on the opposite end had only a small impact on the substrate acceptance. Even stronger negative effects on substrate reduction were observed when the smaller aspartic acid was introduced instead of glutamic acid. We also analyzed the enzyme kinetics of AnMsrA as shown in Fig. (30A and 30B).

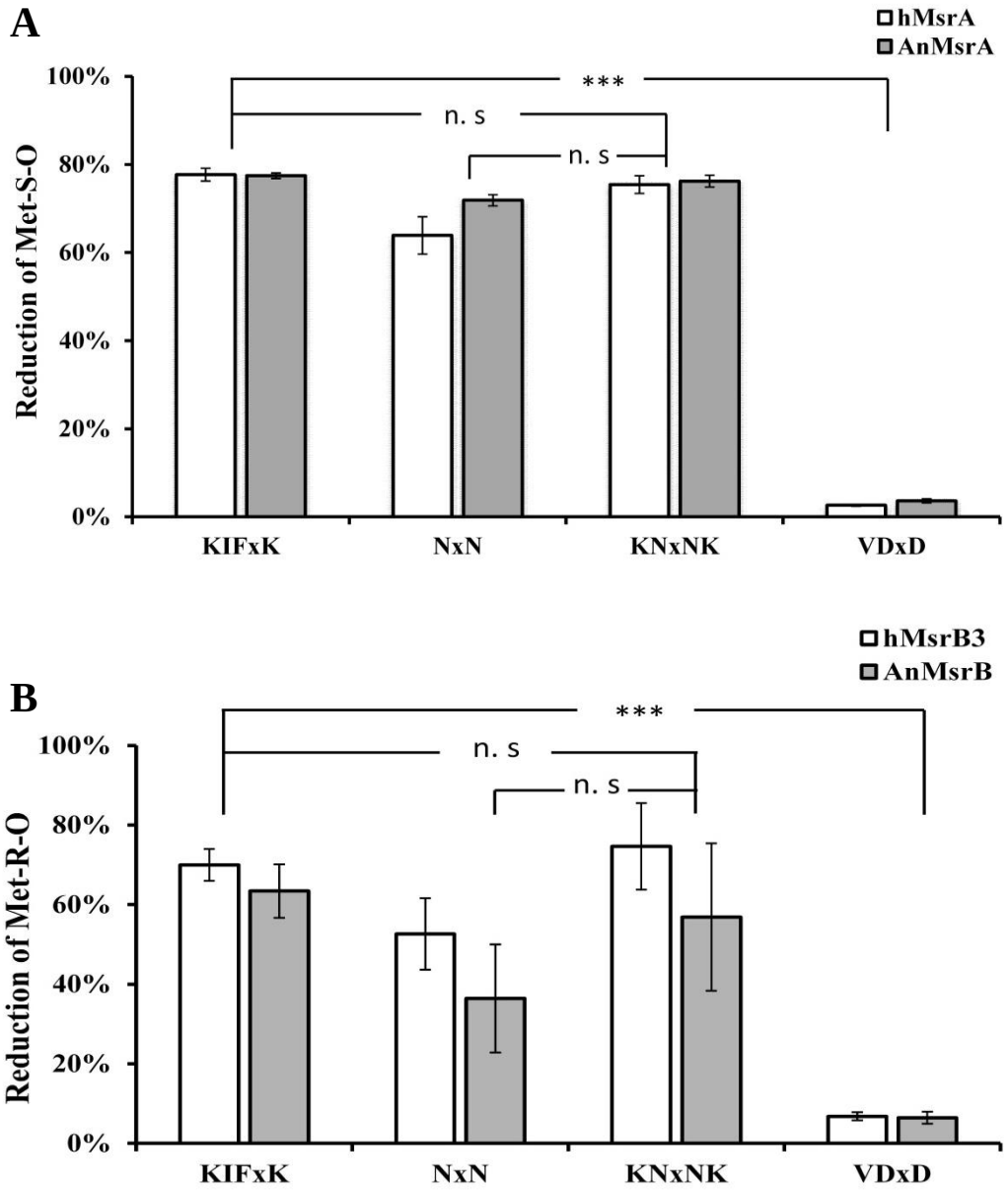

Fig. 29: Influence of the lysine-flanking on the reduction of MetO by MsrAs. Synthetic peptides containing oxidized methionine (mixture of R- and S-isomers) were subjected to reduction by MSR from human and *A. nidulans*. Reactions were carried out with 2 µg MsrA **(A)** or 5 µg MsrB **(B)** and 20 µM peptide for 15 min at 37 °C in the presence of 20 mM DTT and 50 mM Tris/HCl (pH 8). The methionine sulfoxide is indicated with "x". The S. E. was determined from three independent measurements.

The symmetric peptide DM(O)D was almost not converted during 15 min and the asymmetric peptides DM(O)N and NM(O)D had intermediate turnover rates (Fig. 30A). Figure (30B) represents the reaction rate of Met-S-O reduction by AnMsrA. Comparison of reduction rate of the Phe-containing peptides showed that the symmetric peptide (FM(O)F) had a lower reduction rate than the asymmetric peptides (FM(O)K and KM(O)F). Also this comparison

showed that Phe on the right side of MetO (KM(O)F) has no effect on the reduction rate by AnMsrA.

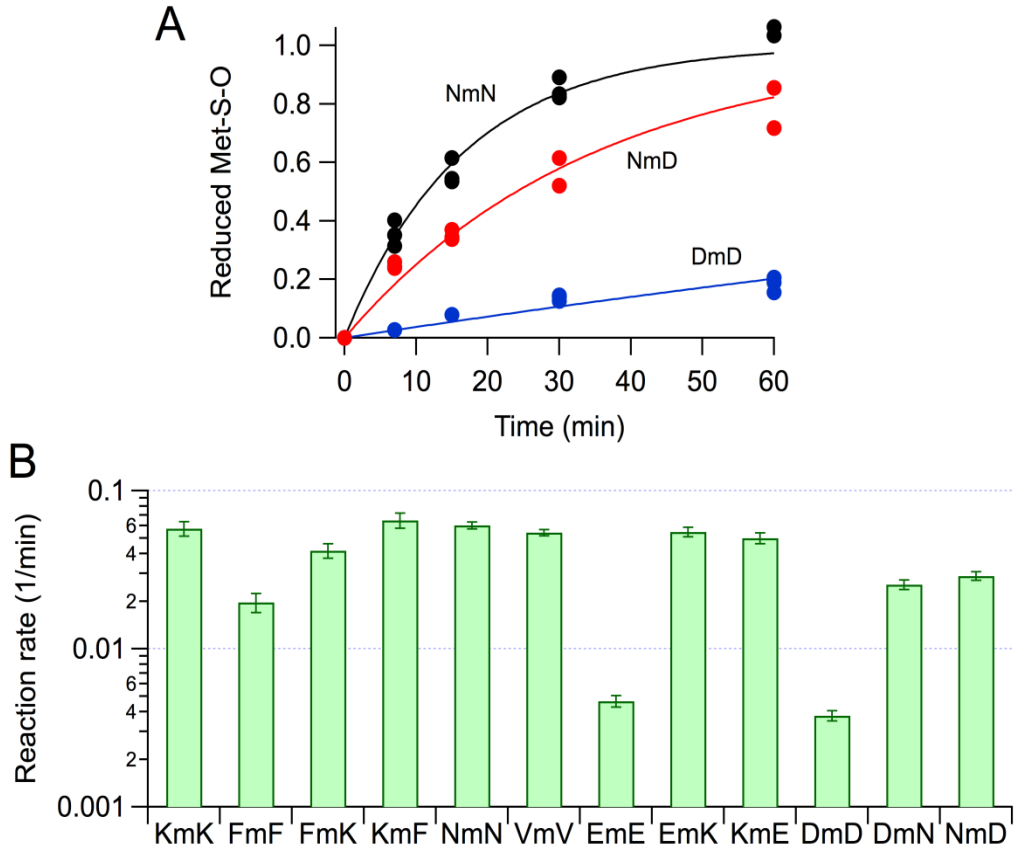

Fig. 30: Kinetic analysis of AnMsrA activity. Reactions were carried out with 2 µg of AnMsrA and 15 µM peptide at 37 °C in the presence of 20 mM DTT and 50 mM Tris/HCl (pH 8.0). **(A)** Time course showing a comparison of Met-S-O reduction in 3 peptides by AnMsrA. **(B)** Reaction rate of Met-S-O reduction by AnMsrA. (m) represents the methionine sulfoxide

3.4.2 Peptide specificity profile of MsrB enzymes

Both types of methionine sulfoxide reductases, MsrA and MsrB share similar catalytic mechanism whereas their structures are very different (Lowther et al., 2002). Thus, peptide sequence preferences of MsrA and MsrB enzymes might differ due to differences in the active site structures. To compare MsrA and MsrB specificities, the same panel of substrate peptides as shown in Fig. (31) was also analyzed for reduction rates by recombinant human MsrB3 and MsrB form *A. nidulans*. As summarized in Fig. (32), the general acceptance of the peptide spectrum resembled the pattern seen with MsrAs, e.g. the positive charged peptide KKM(O)KK had highest conversion rates with both enzymes and the peptide with symmetric aspartate residues (KDM(O)DK) showed reduced conversion.

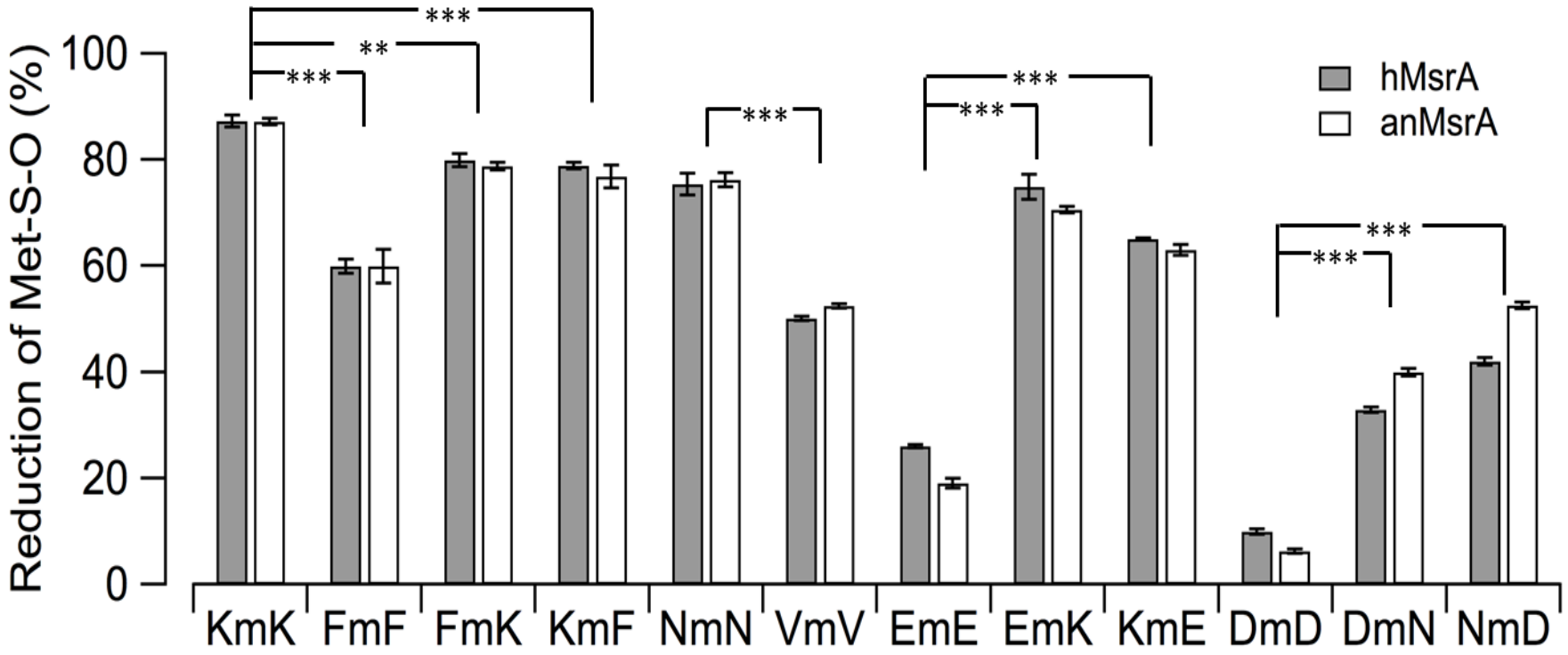

Fig. 31: Influence of the peptide sequence on the reduction of methionine sulfoxide by MsrAs. Synthetic peptides containing oxidized methionine (mixture of R- and S-isomers) were subjected to reduction by methionine sulfoxide reductase from human (hMsrA) and *A. nidulans* (AnMsrA). Reactions were carried out with each 2 µg enzyme 20 µM peptide for 15 min at 37 °C in the presence of 20 mM DTT and 50 mM Tris/HCl (pH 8). The methionine sulfoxide is indicated with "m". The SEM was determined from three independent measurements.

However, in contrast to MsrAs, the influence of the peptide sequence on the reaction with MsrBs was less pronounced than with MsrA enzymes. The peptide with symmetric glutamates had very similar conversion rates as peptides with hydrophobic residues or with asparagine in the flanking positions. The poor substrate KDM(O)DK was still much better reduced with MsrB enzymes than with MsrAs. Interestingly, for both MsrBs the impact of a single aspartate at position -1 was relatively strong with a reduction rate similar to the symmetric peptide (hMsrB3) or even below (AnMsrB). For MsrB from *A. nidulans*, the conversion rates with some, but not all peptides were below the rates found with human MsrB3, indicated small differences in the peptide preferences of both enzymes.

3.4.3 Mutating the active site of AnMsrA and AnMsrB

Three-dimensional structures of several MsrA and MsrB proteins are known and comparison of the active sites of both enzyme classes revealed a mirror-like relationship (Lowther et al., 2002), well in line with the opposite chirality of the respective substrates. All Msr enzymes contain a conserved cysteine (or selenocysteine) in the center of the active site. For MsrA enzymes, two highly conserved negative charges, glutamate and aspartate, are found in the active site cavity and in close vicinity to the cysteine (Fig. 33C). By contrast, the active center of MsrB enzymes does not include negative charges, but positive charges (arginine and histidine) are conserved in most MsrBs (Fig. 33D). Thus, it appears plausible for MsrA proteins that negative charges in the active site pocket cause or at least contribute to the observed low conversion rates of substrate peptides with negative charges flanking Met(O) and the maximal conversion rate of the peptide with the most positive charges (KKM(O)KK). To test this hypothesis, the residues Glu99 and Asp134 in AnMsrA were substituted with positive or polar residues. In a complementary approach, AnMsrB was mutated to replace a positive charge (Arg121) by a neutral or negatively charged residue.

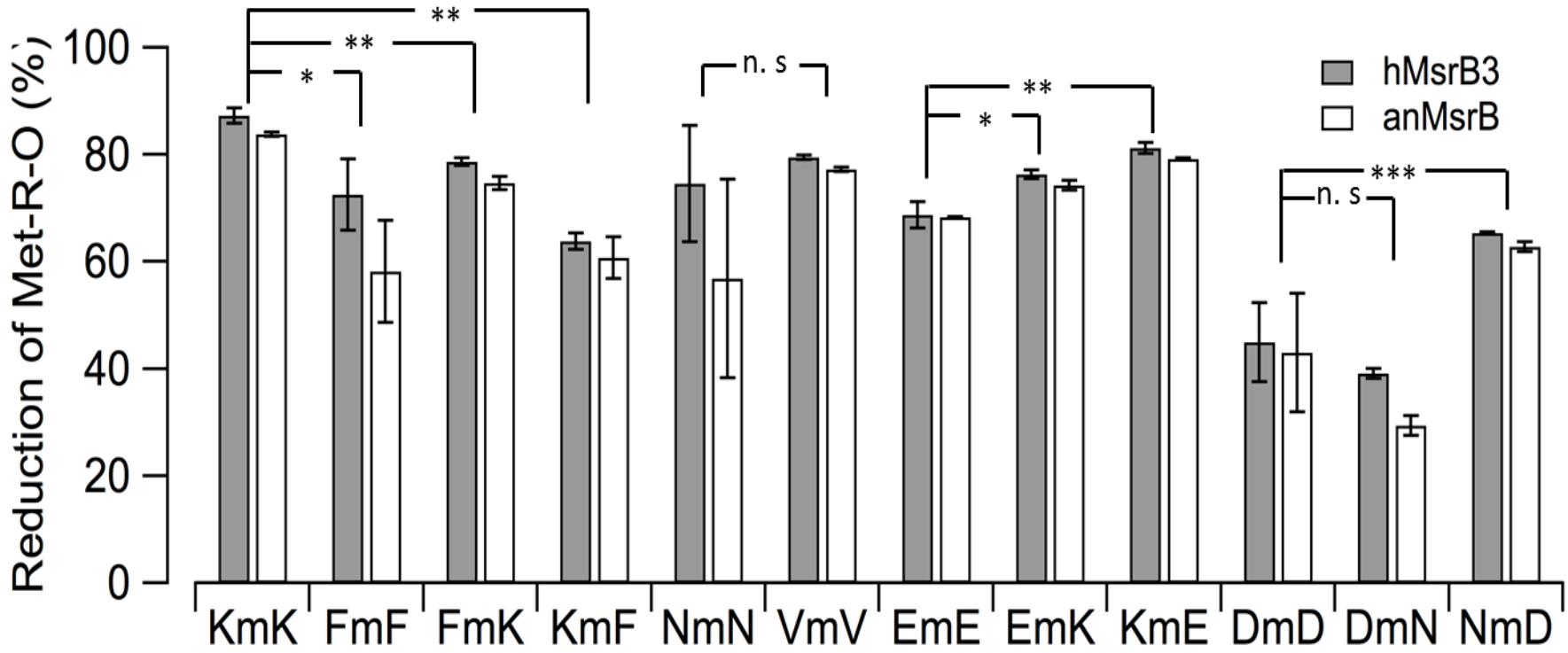

Fig. 32: Influence of the peptide sequence on the reduction of methionine sulfoxide by MsrBs. Synthetic peptides containing oxidized methionine were subjected to reduction by methionine sulfoxide reductase from human (hMsrB3) and *A. nidulans* (AnMsrB). Reactions were carried out with each 5µg enzyme and 20 µM peptide for 15 min at 37 °C in the presence of 20 mM DTT and 50 mM Tris/HCl (pH 8). The methionine sulfoxide is indicated with "m". The SEM was determined from three independent measurements.

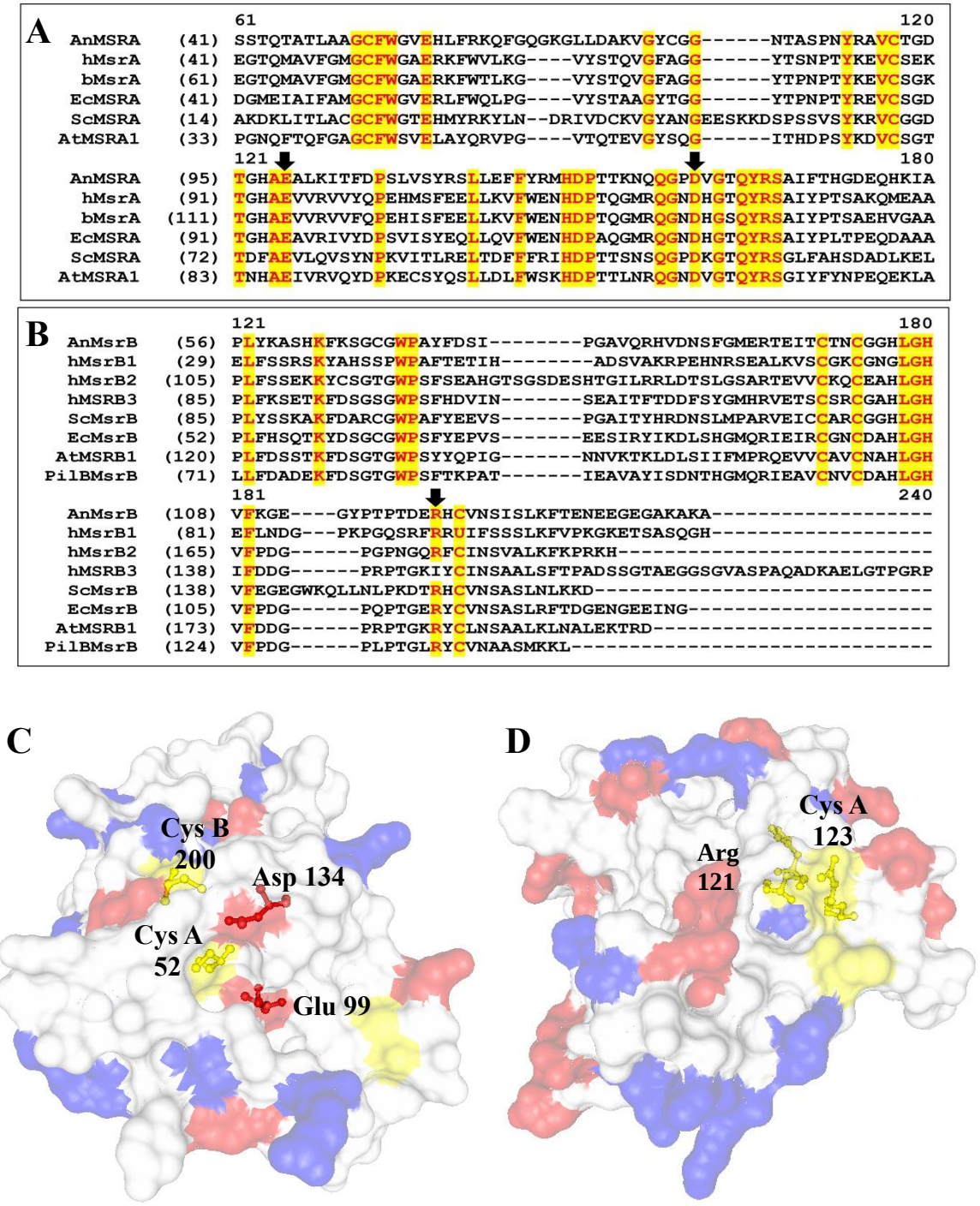

Fig. 33: (A) Multiple sequence alignment of MsrAs proteins, the arrows indicating the conserved acidic residues Glu99 and Asp134. **(B)** Multiple sequence alignment of MsrBs proteins, the arrows indicating the conserved basic residues Arg121. The surface representation of *A. nidulans* MsrA **(C)** and MsrB **(D)** models. The color of the model on the right side as follows; basic residues in blue, acidic residues are in red, yellow represents the Cys residues and white for the rest of the residues. The figure was prepared by using PyMOL (http://www.pymol.org).

3.4.4 Role of Glu99 and Asp134 in AnMsrA active site

To assess their impact on selectivity, the active site charges Glu99 and Asp134 in AnMsrA were mutated and eight mutant constructs were tested for activity. As summarized in Table (3), five constructs were found inactive with all available peptides. These dead mutants included four different replacements of Glu99, while only one mutation of this position (E99Q) was tolerated and the resulting protein had measurable activity. Replacements of this site by Asn or Arg resulted in inactive proteins, as well as the double mutation E99Q•D134N (QN). Replacement of Asp134 by Glu or Asn was tolerated, but introduction of a positive charge (Arg) at this position also resulted in a complete loss of activity. The residual three active mutant proteins were further analyzed for peptide selectivity, using seven different peptides (Fig. 34). Interestingly, the mutant enzyme with Glu replacing Asp134 (termed EE) showed reduced discrimination between the offered substrates and altered order of preference, in comparison to wild type enzyme. Three of the peptides with negative residues in the test positions (-1 or +1) were converted with higher efficiency than peptides with hydrophobic residues (Phe or Val) flanking methionine sulfoxide.

Remarkably, the EE mutant reduced symmetric negative test peptides ((KDM(O)DK, KEM(O)EK) more efficiently than wild-type AnMsrA. Thus, the aspartate in position 134 of the enzyme has a strong impact on selectivity and replacement by glutamate largely removed this selectivity while the overall activity was still high. By contrast, replacement of Glu99 or Asp134 by neutral polar residues resulted in strongly reduced overall activity, but did not principally alter the selectivity in comparison to wild type.

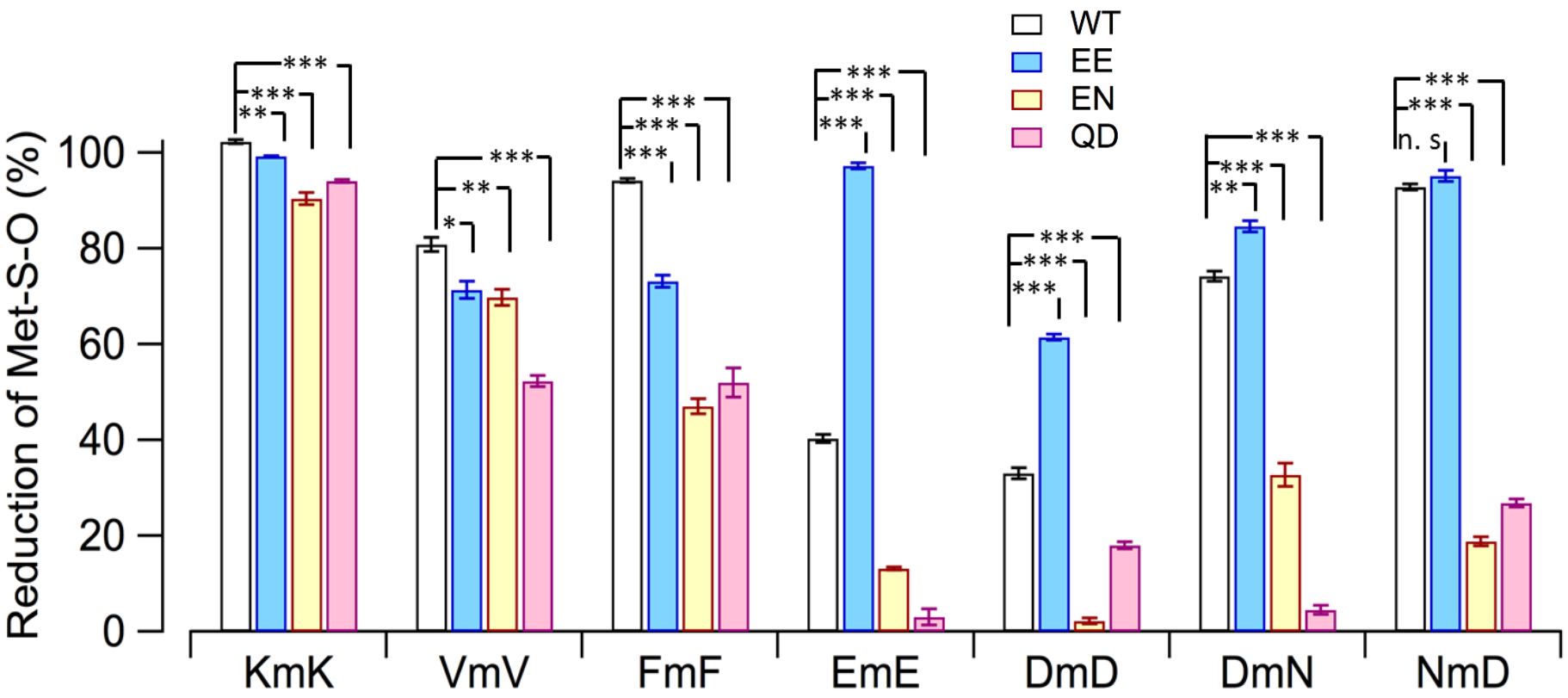

Fig. 34: The activity of the mutated AnMsrA in comparison with the wild type. Synthetic peptides containing oxidized methionine were subjected to reduction by *A. nidulans* MsrA (wt) or the mutated, after the incubation with 20 µg of the recombinant protein and 20 µM peptide for 30 min at 37 °C in the presence of 20 mM DTT and 50 mM Tris (pH 8). The methionine sulfoxide is indicated with "m". The SEM was determined from three independent measurements.

Table 3: Site-directed mutagenesis for the charged residues in the active site of AnMsrA and AnMsrB. The activity was tested toward all the listed peptides. (+++) represents the ability of AnMsr to reduce more than 80 % of MetO in 30 min at 37 °C, and (++) the ability to reduce 50 % of MetO, while (---) represents a less than 10 % reduction of MetO.

Name	Position 99	Position 134	Activity
MsrA			
AnMsrA (wt, ED)	E (Glu)	D (Asp)	+++
AnMsrA (QD)	Q (Gln) (E99Q)	D (Asp)	+
AnMsrA (DD)	D (Asp) (E99D)	D (Asp)	----
AnMsrA (EN)	E (Glu)	N (Asn) (D134N)	+
AnMsrA (EE)	E (Glu)	E (Glu) (D134E)	+++
AnMsrA (DE)	D (Asp) (E99D)	E (Glu) (D134E)	----
AnMsrA (ER)	E (Glu)	R (Arg) (D134R)	----
AnMsrA (RD)	R (Arg) (E99R)	D (Asp)	----
AnMsrA (QN)	Q (Gln) (E99Q)	N (Asn) (D134N)	----
MsrB	**Position 121**		
AnMsrB (wt)	R (Arg)		+++
R141D	D (Asp)		++
R141N	N (Asn)		++

3.4.5 Role of Arg121 in AnMsrB active site

The comparison of peptide selectivity in MsrA and MsrB proteins revealed a weaker discrimination by MsrB enzymes with a better acceptance of substrates with negative charges flanking Met(O) (Figs. 29 and 32). To study the potential impact of surface charges in the active site pocket of MsrB on substrate selectivity, Arg121 in AnMsrB was replaced by a polar (Asn) or negatively charged residue (Asp). Both resulting mutants formed proteins with enzymatic activity, indicating that the positive charge is not crucial for the reaction mechanism. A comparison of these mutants to wild-type AnMsrB was carried out on the same set of seven peptides as in the study of MsrA mutants (Fig. 35). Interestingly, both mutants had a similar impact on substrate discrimination. While the reduction of peptides with positive charge (KKM(O)KK) or hydrophobic residues (KVM(O)VK, KFM(O)FK) was unaffected by the mutations, the reduction of Met(O) flanked by negative charges was less efficient and almost abolished for a peptide with two flanking aspartate residues (KDM(O)DK). These data support the hypothesis that a single residue in the active center of Msr enzymes can determine substrate specificity. Neutralization of the positive charge in position 121 of AnMsrB was already sufficient to induce a strong rejection of peptides with negative charges next to Met(O), similar to the substrate preference seen in MsrA enzymes.

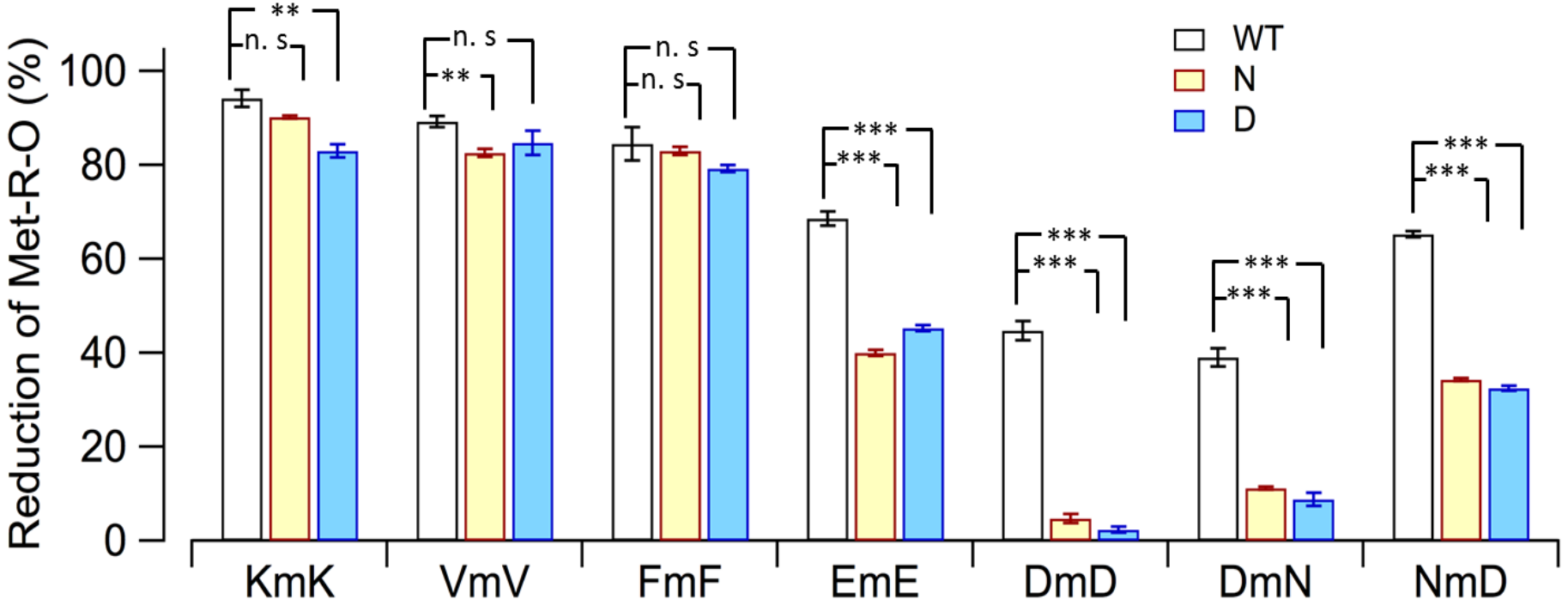

Fig. 35: The activity of the mutated AnMsrB in comparison with the wild type. Synthetic peptides containing oxidized methionine were subjected to reduction by *A. nidulans* MsrB (wt) or the mutated, after the incubation with 20µg of the recombinant protein and 20 µM peptide for 30 min at 37 °C in the presence of 20 mM DTT and 50 mM Tris (pH 8). The methionine sulfoxide is indicated with "m". The SEM was determined from three independent measurements.

4 Discussion

4.1 Methionine sulfoxide reductases in *A. nidulans*

Accumulation of oxidized proteins has been hypothesized to be an important mechanism of the aging process, diabetes and neurodegenerative diseases (Hoshi and Heinemann 2001; Levine and Stadtman 2001; Hou et al., 2002; Moskovitz 2005; Petropoulos and Friguet 2005; Cabreiro et al., 2006; Friguet 2006; Styskal et al., 2012; Styskal et al., 2013). In any living cell, methionine exists in two forms; as a free amino acid or as a residue in proteins and both forms are sensitive to oxidation. Oxidation of Met to MetO creates two epimers (S and R), which are substrates for specific reductases (MSR). In all studied organisms, the *S*-epimer requires one type of MSR enzyme, MsrA, which is responsible for the reduction of free Met-S-O and of bound Met-S-O. In contrast, the reduction of R-epimers requires two types of enzymes; one type to repair the free Met-R-O (fRMsr) and another one for the protein-bound Met-R-O (MsrB). While these enzymes are well-characterized structurally and functionally and their critical roles in the repair of oxidized Met residues in proteins have been established, it is still unclear how free Met-O is reduced by multicellular organisms. MsrA does show activity toward free Met-S-O, but the activity of MsrB toward free Met-R-SO is low and fRmsr enzymes have not yet been found in higher organisms (Kim and Gladyshev 2007; Lee et al., 2009).

Recently, Lin and his co-workers (2007) and Le and his co-workers (2009) identified in *E. coli* and *S. cerevisiae*, respectively, a specialized type of Msr that only reduce free Met-R-O, designated fRMsr. Comparative genomics analysis performed by Le and his co-workers (2009) of fRMsr homologs in sequenced genomes showed a mosaic distribution of this protein. Their studies suggested that fRMsr is absent from all multicellular organisms, including higher plants and animals.

Our data showed the presence of a fRMsr homologos protein in the filamentous-fungus *A. nidulans* (accession number XP_680937). This protein shares 85-88 % similarity with other hypothetical proteins in *Aspergillus* species such as *A. terreus*, *A. nigr* and *A. oryzae*. The occurrence of fRMsr in multicellular organisms like fungi raises the possibility of its presence in even higher organisms. We decided to study these enzymes in a filamentous fungus representing a multicellular organism which is more complicated than *E. coli* and yeast. This study describes the characterization of three methionine sulfoxide reductases (AnMsrA, AnMsrB and AnfRMsr) from the filamentous fungus *A. nidulans*. The sequences and the *in*

vitro characterization of the recombinant proteins; specific activity, substrate specificity, biochemical properties and catalytic mechanism of each enzyme were determined. Furthermore, knockout strains for the three enzymes were compared to wild-type under oxidative stress conditions.

4.2 The biological significance of Msrs for *A. nidulans*

The single knockout strains showed that lacking any of *msr* genes has a drastic influence on the morphology of the fungus. Interestingly, Δ*msrB* showed slower growth rate compared with the wild type and the other knockouts. While this result is in agreement with the data from Soriani et al. (2009), is as opposed to the data collected from yeast (Koc et al., 2004; Le et al., 2009). The Δ*msrB* showed a clear delay in the radial growth suggesting that MsrB might has another important role in the regulation of the growth on solid media for *A. nidulans*.

4.2.1 Sensitivity of *msr* knockouts to oxidative stress

The response of *A. nidulans* lacking *msr* genes to three different oxidants was clarified. Although all these compounds cause oxidative stress, their respective effects might be different. While the *fRmsr* knockout showed less sensitivity toward all of the oxidative stressors compared with *msrA* and *msrB* knock outs, its sensitivity was higher than the wild type. fRMsr was characterized biochemically and structurally from *E. coli* (Lin et al., 2007), yeast (Le et al., 2009) and *N. meningitides* (Gruez et al., 2010), but the lack of fRmsr was only studied in yeast (Le et al., 2009). In agreement with our results, deficiency of fRMsr induced less sensitivity than Δ*msrA* or Δ*msrB*. Since fRMsr is strictly specific for the R-epimer of the free methionine, one may speculate that oxidation of free Met-O has a less deteriorating impact on the stress resistance than oxidation of MetO in proteins.

MsrA was studied well before MsrB, so that the majority of the studies in the literature were performed with MsrA. Lacking MsrA caused increased susceptibility to oxidative stress in mice (Moskovitz et al., 2001), yeast (Moskovitz et al., 1997; Le et al., 2009), and bacteria (John et al., 2001; Dougles et al., 2004). Surprisingly, deleting MsrB from *A. nidulans* aroused more sensitivity to H_2O_2 and mendione than deleting MsrA. In contrast to our results, all of the previous studies showed that Δ*msrA* is more sensitive to oxidative stress than Δ*msrB* (*i.e.* bacteria, Dhandayuthapani et al., 2009; Denkel et al., 2011; yeast, Le et al., 2009; fruit fly, Shchedrina et al., 2009). The Δ*msrB* strain was hypersensitive to menadione even at the

lowest concentration used (10 mM) its growth was almost inhibited. The possibility of diastereoselective protein methionine oxidation was shown by Sharov and Schöneich (2000) who found that diastereoselectivity is dependent on the nature of the oxidizing agent. This result would be expected if menadione had a preference to generate the R-epimer of MetO.

4.2.2 Antioxidant role of fungal Msrs

The expression levels of *msrA*, *msrB* and *fRmsr* mRNA in *A. nidulans* wt strain TNO2a3 were examined under oxidative stress generated by H_2O_2, Ch-T and paraquat. Paraquat has two potentially important consequences relevant to the development of toxicity in biological systems: generation of ROS and/or depletion of cellular reducing equivalents (e.g., NADPH) necessary for normal function (Bus and Gibson, 1984). The qRT-PCR analysis revealed that under normal conditions, *msrA* was strongly expressed in the mycelia followed by *fRmsr* whereas *msrB* was less expressed by at least 20-fold compared to *msrA*. The RT-PCR analyses also showed increase in the expression of *msrA*, *MsrB* and *fRmsr* in response to oxidative stress caused by the above oxidants. In contrary to our results, a study on *S. Aureus* which harbors 3 *msrA* genes and one *msrB* gene foundd that under oxidative, alkaline, acidic or osmatic stress conditions none of these genes showed any significant change in their expression, except for *msrA2*, which was repressed by acidic pH conditions and *msrA3* showing 5.5-fold increase under osmotic stress conditions (Singh and Singh, 2012). Similarly microarray analysis showed that the expression of *msrB* gene did not change under the condition of sulfur limitation in yeast cells grown aerobically (Le et al., 2009). Other oxidants have been tested by Singh and Singh (2012) such as cumene hydroperoxide, methyl viologen, diamide, and N-ethylmaleimide (NEM), repressed the expression of the *msr* genes to a significant level.

The observed upregulation of the three *msr* genes suggested that all Msr enzymes are involved in stress response in *A. nidulans*. These data are in the line with the finding that the lack of Msr enzymes in knockouts affected the stress resistance in inhibition zone assays. Taken the data from the inhibition zone and the gene expression together the impact of MsrB on stress response in *A. nidulans* appears stronger than the two other *msr* genes, MsrA and fRMsr. This is in contrast to studies on other organisms (*i.e.* bacteria, Dhandayuthapani et al., 2009; Denkel et al., 2011; yeast, Le et al., 2009; fruit fly, Shchedrina et al., 2009). We propose that the reason for that could be: (i) the main and the only enzyme responsible for repairing bound Met-R-O in *A. nidulans*. (ii) The delay in growth and the hypersensitivity of

Δ*msrB* and suggesting that MsrB might be critically involved in early steps of fungal growth and/or signal transduction. (iii) The significance of MsrB for *A. nidulans* might be due to that the R-epimer of bound MetO is more frequent or more significant than S-epimer.

4.3 Characterization of substrate specificity of fungal Msrs

The specificity of recombinantly purified *A. nidulans* MsrA, MsrB and fRMsr was determined using free MetO and a synthetic peptide containing MetO (Ac-KIFM(O)K-Dnp). The data confirmed that MsrA is able to reduce free Met-S-O as well as peptide bound Met-S-O. It was also confirmed that MsrB can reduce peptide-pound Met-R-O, but has very low activity toward free Met-R-O. In contrary to MsrB, fRMsr showed high activity toward reduction of free Met-R-O and inability toward reduction of peptide-bound Met-R-O. Human MSRs were used as a positive control for the *A. nidulans* MSRs in the enzymatic activity and biochemical characterization. The enzymatic activity for both human and fungal recombinant MSRs showed a maximum activity at pH 8.0 and almost no activity at pH 6.0 and below. In agreement with my data, the optimum pH for tomato (*Solanum lycopersicum*) MsrA2 activity was reported to be at pH 8.0 and also this enzyme showed no activity below pH 6.0 (Dia et al., 2011). In contrary, bacterial MsrA from *C. elegans* showed the highest level of activity between pH 7.0 and 7.5 and its activity significantly decreased below pH 6.5 and above pH 8.5 (Lee et al., 2005).

4.4 Identification of catalytic Cys in *A. nidulans* Msr

The catalytic Cys and the active site for all three types of MSR enzymes have been identified for different organisms (i.e. hMsrA, Hansel et al., 2002; hMsrB, Kim and Gladyshev 2004; yeast fRMsr, Le et al., 2009). The catalytic mechanisms differ between different Msrs, especially in the number of recycling cysteines. Hence, it was important to determine the catalytic Cys for *A. nidulans* MSR. For all MSRs, the catalytic mechanism is based on three steps. In the first step, a catalytic Cys (CysA) residue interacts with the methionine sulfoxide, leading to Met release and the formation of a sulfenic acid in the enzyme. The second step involves the formation of a disulfide bridge between CysA and a restoring Cys (CysB). In the final step, CysC attacks CysB of the CysA-CysB disulfide bond to form a new CysB-CysC disulfide bond. This disulfide bridge is then reduced by an electron donor, the NADPH-dependent Trx/TrxR system, leading to the recovery of the Msr active site (Lowther et al., 2000; Neiers et al., 2004; Kim and Gladyshev 2007; Ugarte et al., 2010; Sreekumar et al., 2011).

4.4.1 Methionine sulfoxide reductase A

There are three types of MsrA enzymes classified based on the number of Cys that are involved in the reduction of Met-S-O. Group I requires 3 Cys; the CysA which is located in a GCFWG motif and for recovery, CysB and CysC are required which, typically separated by a Gly rich region, e.g. in hMsrA and *E. coli* MsrA (Kauffmann et al., 2005). Group II; MsrAs of this group do not require a CysC such as *M. tuberculosis* MsrA, a CysB belongs to a GYCH or GYxCH motif. In group III, also two Cys are involved in the catalysis of Met-S-O but the difference in this group is CysA and CysB are in the same motif (GCFWC) such as in MsrA of *Bacillus subtilis* (Kauffmann et al., 2005).

Using site directed mutagenesis, all Cys residues in *A. nidulans* MsrA were mutated individually in order to identify the catalytic mechanism of AnMsrA. The data showed that the activity of AnMsrA was only affected by mutated Cys52 and Cys200 whereas no effects of mutating Cys78 or Cys91 on the activity were observed. These results located the *A. nidulans* MsrA in group II in which only two Cys are involved in the catalytic mechanism, CysA belongs to motif GCFWG while CysB is found in a GYCH motif. The functional identification of Cys52 in *A. nidulans* MsrA is based on the finding that this mutant showed no activity when DTT was used as an electron donor. By contrast, mutant Cys200Ser was active under these conditions, but inactive when Trx system was used for enzyme recovery. Substitution of Cys52 clearly inactivated the enzyme with both reductant systems suggesting that Cys52 is the only candidate to be the catalytic Cys (CysA). DTT is able to react directly on the CysA and reduce the sulfenic acid whereas Trx requires a disulfide bond between CysA and CysB. Figure (34A) represents a homology model for AnMsrA based on the known structure of *Mycobacterium tuberculosis* MsrA (PDB: 1NWA). A proposed mechanism for the catalysis of Met-S-O by AnMsrA is shown in Fig. (34B). Where AnMsrA reduces Met-S-O through CysA (Cys52). In the presence of DTT, the enzyme can be directly recovered, while under physiological conditions a disulfide bond would be formed internally between CysA and CysB (Cys200). Then the disulfide bond would be reduced by thioredoxin (Trx) using a thiol-exchange reaction. This model is well in line with the location of the cysteine residues in the structure model (Fig. 34A). While CysA and CysB are in close enough proximity to form a disulfide, the other two cysteines are located further outside the active center cavity.

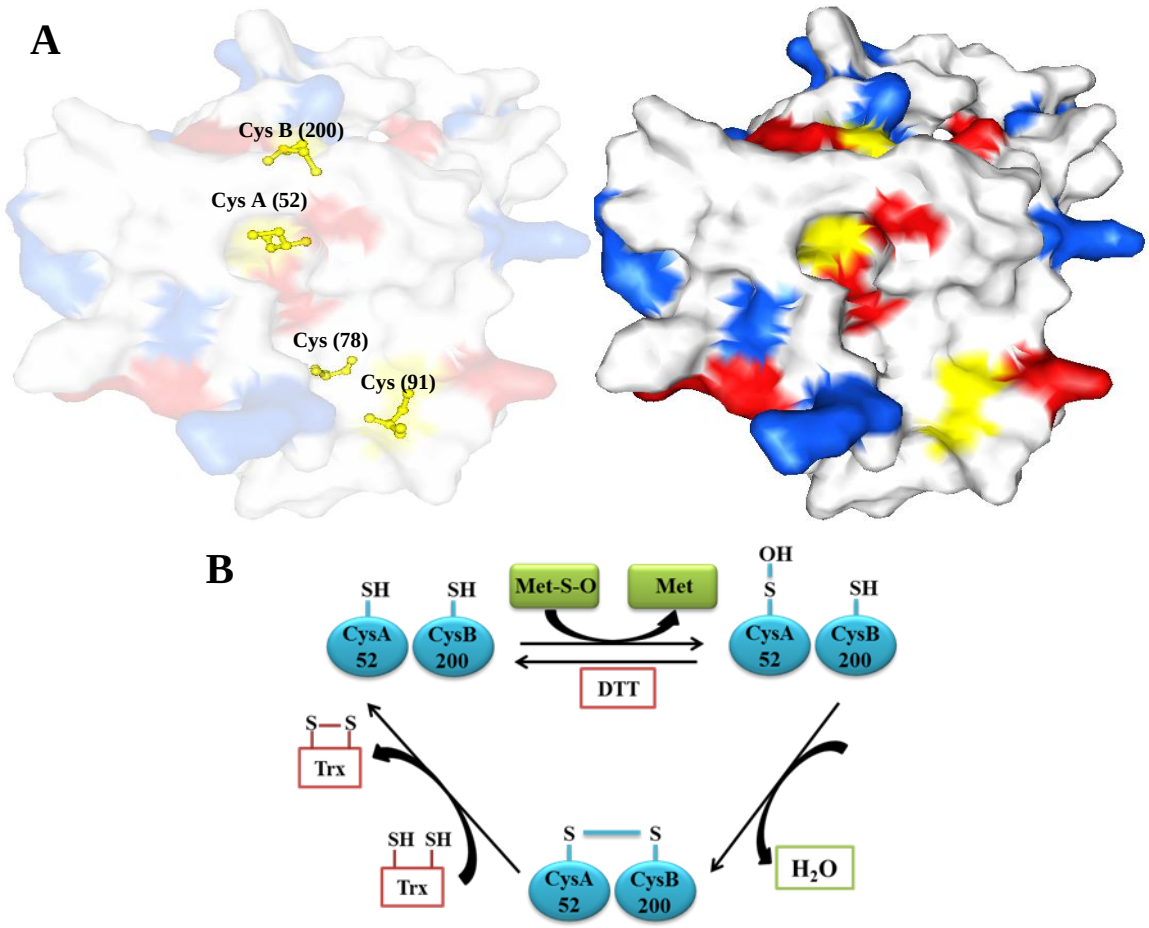

Fig. 34: A model for the catalytic mechanism of *A. nidulans* MsrA. (A) Surface representation of the 3D structure model which was made by using the SWISS-MODEL workspace (http://swissmodel.expasy.org/workspace/). All the Cys residues are shown on the model on the left side. The color of the model on the right side as follows; basic residues in blue, acidic residues are in red, yellow is representing the Cys residues and white for the rest of the residues. The figure was prepared by using PyMOL (http://www.pymol.org). **(B)** A proposed catalytic mechanism for *A. nidulans* MsrA.

4.4.2 Methionine sulfoxide reductase B

Based on the catalytic mechanism and the coordination of zinc, the evolutionary divergence of MsrB proteins resulted in two major groups. While in group I, MsrB is a zinc-dependent enzyme and require only one Cys for the reduction of Met-R-O, MsrB of group II lacks the zinc-binding site and requires a recovery Cys that forms a disulfide bond with the catalytic Cys (Kumer et al., 2002). Taken together, AnMsrB lacks the recovery Cys, and contains a zinc-coordination motif and any Cys replacement in this motif inactivated the enzyme. These results located *A. nidulans* MsrB in group I of MsrBs enzymes. The catalytic mechanism for bound-Met-R-O reduction by AnMsrB is represents in Figure (35B). In this mechanism, reduction of Met-R-O is proposed to be achieved in two steps. AnMsrB reduces bound-Met-

R-O in the first step. A result of that step AnMsrB become oxidized and subsequently recovered by DTT (in vitro) or Trx and/or thionein (in vivo).

Kumar and others (2002) demonstrated that zinc is essential for the catalytic function of *Drosophila* MsrB1 and that it was coordinated in the enzyme through four cysteine residues organized in two-CxxC pair motif. Similarly, Kim and Gladyshev (2004) confirmed that all three mammalian MsrBs are Zn-containing proteins. Expectedly, the alignment data showed that this motif is also conserved in *A. nidulans* MsrB. A three-dimensional model of AnMsrB is shown in Figure (35A); it was created based on the MsrB structure from *Xanthomonas campestris* (PDB: 3HCJ). In this model, four cysteines arranged together to form a binding-site for the zinc coordination (Fig. 35A). In agreement with Kryukov and his coworkers (2002), substitution of at least one Cys at this motif completely inactivated the enzyme activity, confirming their important role for the AnMsrB activity and structure stability.

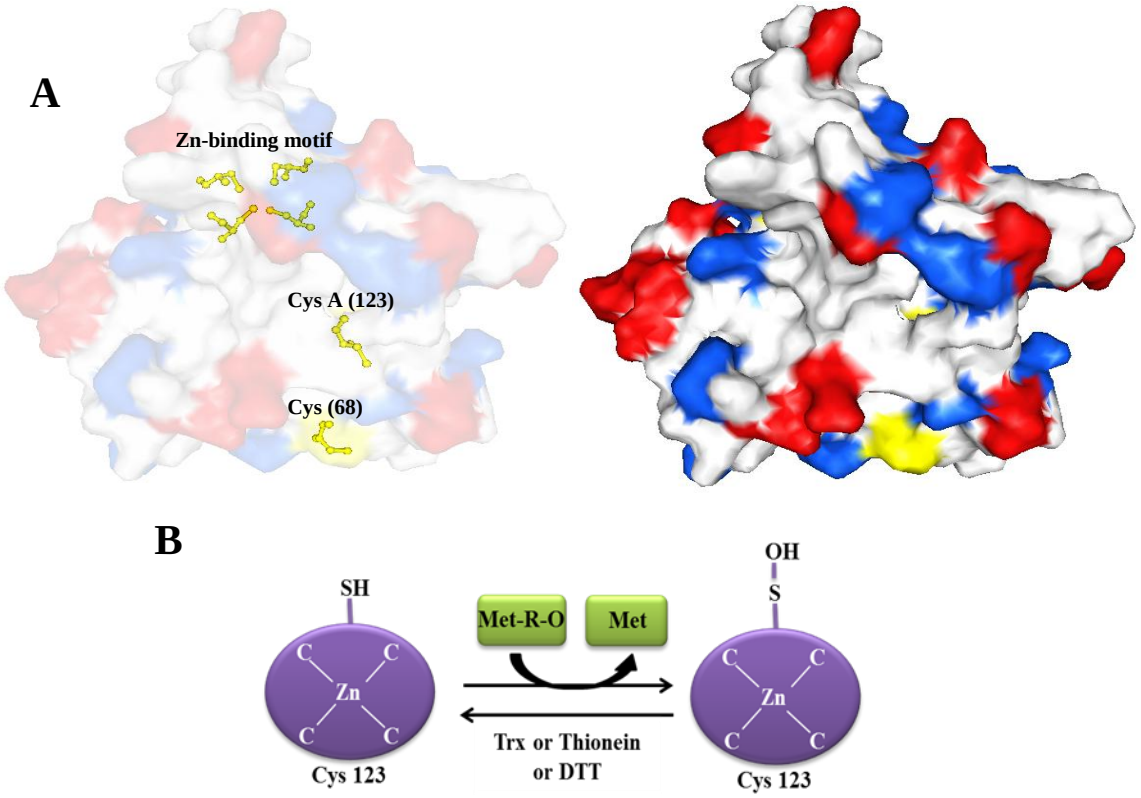

Fig. 35: A model for the catalytic mechanism of *A. nidulans* MsrB (A) Surface representation of the 3D model structure which was made by using the SWISS-MODEL workspace (http://swissmodel.expasy.org/workspace/). All the Cys residues are shown on the model on the left side. The color of the model on the right side as follows; basic residues in blue, acidic residues are in red, yellow is representing the Cys residues and white for the rest of the residues. The figure was prepared by using PyMOL (http://www.pymol.org). **(B)** A proposed catalytic mechanism of *A. nidulans* MsrB.

As shown in the 3D model, AnMsrB has six cysteine residues; four of them are the zinc-binding motif that leaves two candidates for the CysA. As mentioned above, Trx requires a disulfide bridge in order to reduce the oxidized Msr. The substitution of Cys123 in AnMsrB resulted in a complete loss of activity using DTT or Trx as an electron donor suggesting that Cys123 is the catalytic Cys (CysA). The catalytic Cys (Cys123) of AnMsrB is located in the C-terminus and it is the conserved Cys to the Selenocysteine (U95) in hMsrB1. It was reported that Trx might be not the biological reducing system for mammalian MsrB2 and MsrB3 which seems clear from the low activity of both enzymes compared with their activity using DTT (Kim and Gladyshev 2005; Sagher et al., 2006). A heat-stable protein has been identified as a zinc-containing metallothionein (Zn-MT). This thionein (T), which is formed when the zinc is removed from Zn-MT, can function as a reducing system for the Msr proteins because of its high content of cysteine residues and that Trx can reduce oxidized T (Sagher et al., 2006).

4.4.3 Free methionine R-sulfoxide reductase

Studies on the catalytic mechanism of yeast and bacterial fRMsr revealed that both enzymes require three cysteines for MetO reduction (Lin et al., 2007; Le et al., 2009; Kwek et al., 2010). In AnfRMsr, three cysteine residues (positions 104, 114 and 138) are conserved in comparison to yeast and bacterial fRMsr. We mutated these three conserved Cys and found that only two of them (positions 104 and 138) are crucial for the enzyme activity. A 3D structure model of AnfRMsr was made based on the sequence of *Saccharomyces cerevisiae* fRMsr (PDB: 1F5M) (Fig. 36A). The catalytic Cys has been identified at position 114 (in AnfRMsr) for the bacterial and the yeast enzyme by Lin et al., (2007) and Le et al., (2009) respectively. Interestingly, for the fungal fRMsr substitution of this Cys by Ser had no effect on the enzyme activity. In agreement with this results, that Cys114 had no role in free Met_ catalysis were obtained for *S. aureus* fRMsr by Bong et al., (2010). However, in contrast to Le and his coworkers (2009), another group has identified Cys 138 (in AnfRMsr) as the catalytic Cys for yeast fRMsr (Kwek et al., 2010) and for *S. aureus* fRMsr (Bong et al., 2010). Figure (36B) shows a three-steps model proposes a catalytic mechanism of AnfRMsr based on the Cys mutation data. In a first step, Cys138 (equivalent to Cys125 ScfRMsr CysA) reduces free Met-R-O, results in sulfenic acid formation on that Cys. In the second step, the sulfenic acid internally interacts with Cys104 (recycling Cys) to form a disulfide bond. Subsequently, the disulfide bond is then reduced by Trx.

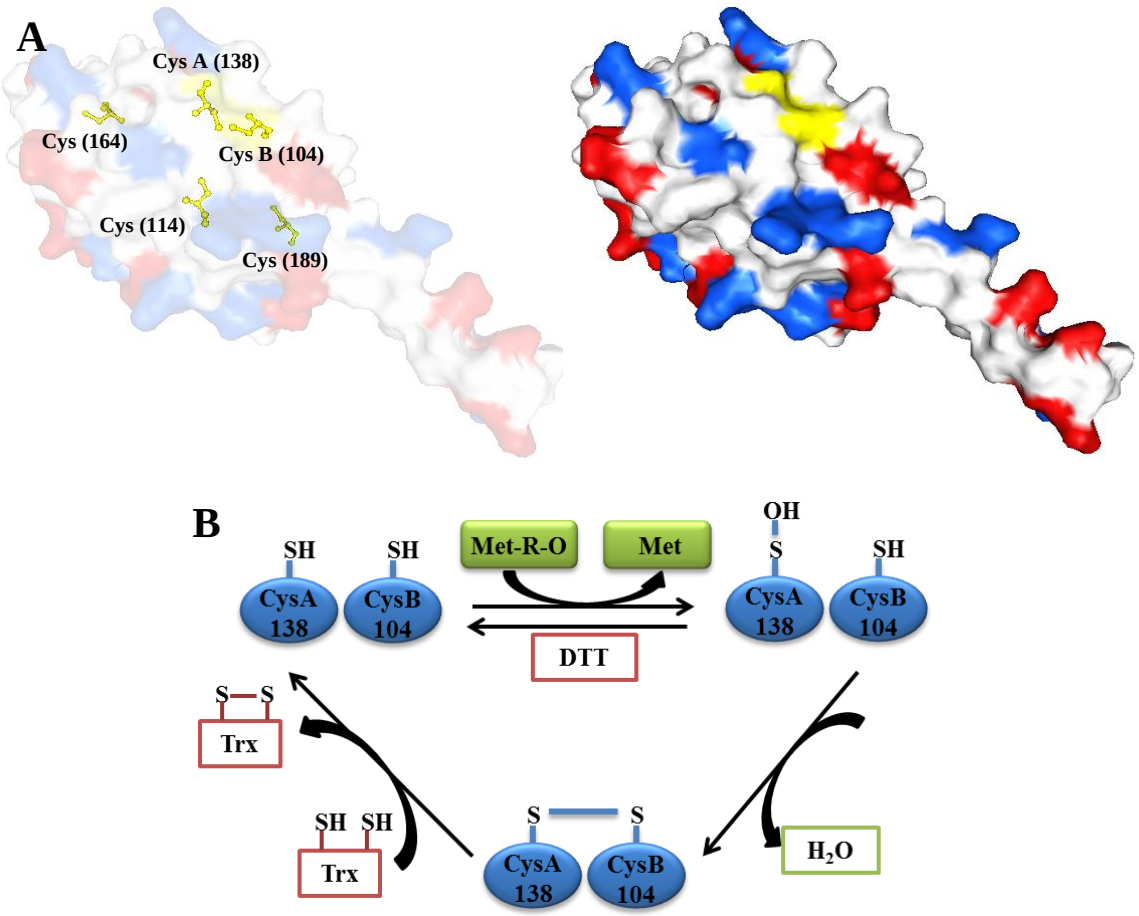

Fig. 36: A model for the catalytic mechanism of *A. nidulans* fRMsr. (A) Surface representation 3D model structure of fRMsr from *A. nidulans*. The homology model structure was made by using the SWISS-MODEL workspace (http://swissmodel.expasy.org/workspace/). All the Cys are shown on the model on the left side. The color of the model on the right side as follows; basic residues in blue, acidic residues are in red, yellow is representing the Cys residues and white for the rest of the residues. The figure was prepared by using PyMOL (http://www.pymol.org). **(B)** A proposed catalytic mechanism of *A. nidulans* fRMsr.

Based on the presence of the three conserved Cys (Cys104, Cys114 and Cys138), fRMsr enzymes exists in two variants. The only difference between these two variants is that variant 2 lacks Cys104 (Le et al., 2009). Comparing different sequences of fRMsr protein revealed that the total number of cysteines exist in each protein is varied among them (Fig. 8). While yeast and fungal fRMsr shares these cysteines residues, they possess different catalytic mechanism. Thus our study suggest that based on the number of Cys residues are involved in the catalysis of free Met-R-O, fRMsr can be exist in two subgroups. Group I contains the enzymes that use three Cys in their catalytis mechanism such as in yeast fRMsr. Group II include the enzymes that have only two Cys involved in the catalysis of free Met-R-O such as in *A. nidulans* fRMsr.

4.5 Peptide specificity of MsrA and MsrB

Theoretically, MsrA and MsrB might have the ability to repair any damaged-protein or regulated by the oxidation of surface-exposed methionine. However, some studies showed that Msr enzymes are selective not only for MetO enantiomers but also for their protein substrate. For example, MsrA and/or MsrB effectively repaired many proteins such as the insulin receptor InR (Styskal et al., 2013), human potassium channel hERG1 (Su et al., 2007), and ShC/B channels (Ciorba et al., 1997), while Msr were not able to repair the methioinine-oxidized apoC-II amyloid fibrils (Binger et al., 2010) or the sodium channel Nav1.4 (Kassmann et al., 2008). These examples raise many questions such as how selective these enzymes are and what mechanism these enzymes use to selectively reduce some substrates over other. In order to address these questions, we first tried to find out what is already known about the protein specificity of Msr. Interestingly, while many scientists have shown interest for studying these group of enzymes at many levels since 1981 (first report was made by Brot et al., 1981), we did not find any article addressing the Msr specificity except for one article by Sun et al., (1999). Sun and his coworkers (1999) reported that repair of oxidized CaM by bMsrA depends on the location of the oxidized methionine with a clear preference for hydrophobic context. Their study showed that the peptide (ELGTVM36R) is a good substrate, while the peptide (LTDEEVDEM124IR) was poorly reduced by bMsrA suggesting that was due to the hydrophobicity of each peptide. Comparing these two peptides, it seems clear that hydrophobicity is not the only difference between them. While the peptide, which was reduced well by bMsrA, has more uncharged residues the other peptide consists mostly from negatively-charged residues. Thus, the charge of the neighboring residues to MetO might have the higher impact on the reduction of MetO by bMsrA. In order to investigate this possibility, three peptides have been designed based on the sequence of the well-reduced peptide proposed by Sun et al., (1999) and the ability of hMsrA to repair them was examined. These peptides have been synthesized to carry a pre-oxidized methionine.

The results showed that hMsrA exhibited higher activity toward a Val-containing peptide than to an Asp- or Arg-containing peptide, supporting the results by Sun et al., (1999). More interestingly, our data revealed that hMsrA showed less activity toward the Asp-containing peptide than Arg-containing peptide suggesting that hydrophobicity is not the only factor in hMsrA selectivity. To further investigate that, a set of peptides differing by single residues based on the physical and the chemical properties of the R group was synthesized and tested. The impact of acidic residues such as Asp and Glu, basic residues such as Lys, aromatic

residues like Phe, hydrophobic residues like Val, and hydrophilic residues such as Asn were examined on the reduction of MetO by Msrs. Interestingly, the results showed a different pattern for both Msr types; MsrAs were strongly impaired by acidic residues flanking to MetO, whereas MsrBs were less sensitive to the flanking amino acids. The data revealed that reduction of MetO is indeed influenced by surrounding residues of Methionine. Interestingly, oxidation of methionine residues in the inactivation motif of the sodium channel Nav1.4 could not be recovered by hMsrA (Kassmann et al., 2008).

Rat, ^{1301}**KDIFMTEEQKK**^{1311}
Human, ^{1308}**KDIFMTEEQKK**^{1318}

There might be two possibilities to explain that; the first possibility is the accessibility of the oxidized Met to hMsrA. The other possibility is that hMsrA was impaired by the acidic residues which surrounded the methionine. The tested peptides in this study were designed examine the ability of MsrAs and MsrBs to reduce MetO in a different context. As the data showed that the presence of MetO in an acidic context clearly transforms it to inaccessible substrate to MsrAs. These results raise a question about whether the tested amino acids occurred in the known proteins. A search of the UniProt database revealed that methionine in protein can often be found in a context with negative charges (Fig. 37). One might speculate that repair of oxidized proteins is dependent on the amino acids neighbors of MetO. Our results suggesting that these data might be used to build a prediction search-tool for Msr possibly-targeted proteins.

4.6 Role of Glu99 and Asp134 in the active site of AnMsrA

To address the mechanism underlying the difference in peptide selectivity of AnMsrA and AnMsrB, we compared their active sites. Although, the crystal structure of MsrB shows no resemblance to MsrA structure, the active sites in both show approximate symmetry (Lowther et al., 2000). The three-dimensional structures of the MsrA from *Escherichia coli*, *Bos taurus,* and *Mycobacterium tuberculosis* have been obtained by x-ray crystallography (Lowther et al., 2000; Tête-Favier et al., 2000; Antione et al., 2003; Olry et al., 2004; Taylor et al., 2003). In all the structures, a water molecule is present, in a position that can mimic the oxygen atom of the sulfoxide function of MetSO. This water molecule found to be tightly H-bonded to three invariant residues Tyr87, Glu94, and Tyr139.

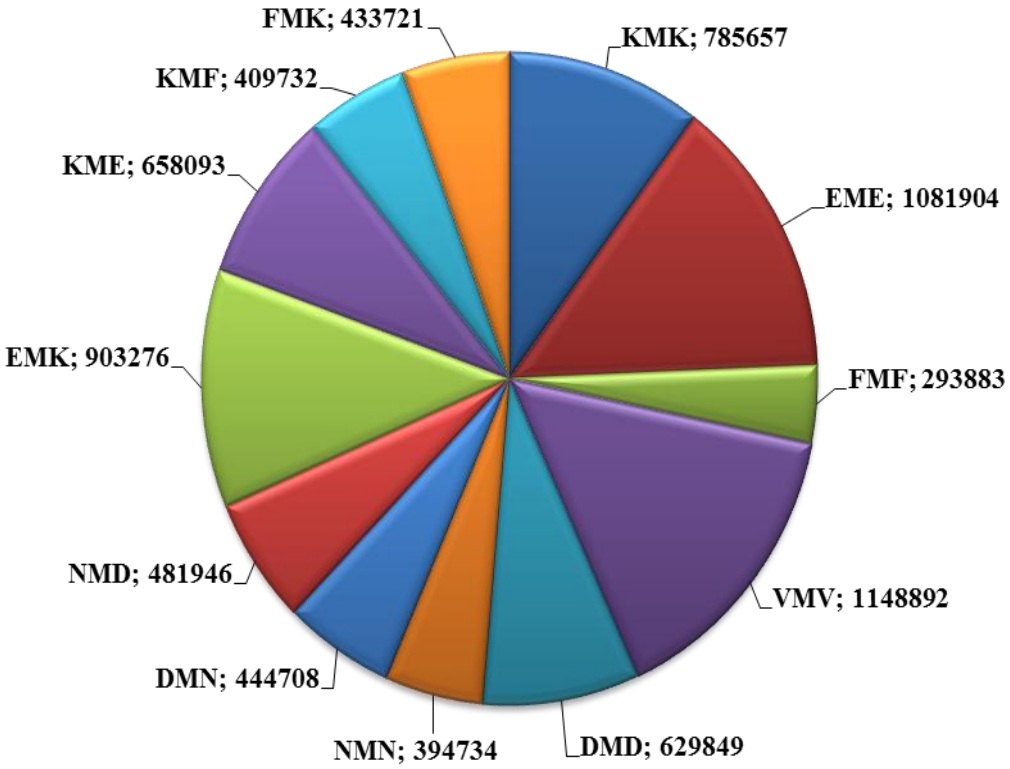

Fig. 37. Occurrence of the designed peptide in the protein database. UniProtKB database was used to align our designed peptides to the known proteins (http://pir.georgetown.edu/pirwww/index.shtml). The numbers represent the repeats of each sequence among the known proteins.

It was also found that Phe53, Asp134, and Trp54 residues were involved in the substrate recognition via the formation of a hydrophobic pocket in which the methyl group of MetSO can bind. Figure (38) shows the active site of *A. nidulans* MsrA and MsrB. Despite the mirror-like relationship between MsrA and MsrB, it is clear from the structure features that MsrBs lacks the acidic residues (Asp134 and Glu99 in AnMsrA) in the active site and instead it have basic residues (Arg121, His104 and His107 in AnMsrB). In order to investigate whether these residues have a role in MetO reduction by AnMsrA or AnMsrB, several point mutations were constructed. Substitution of this residue for Asp or Arg resulted in complete loss of activity while replacement by Gln was tolerated. Antoine and others (2006) suggested that Glu99 (numbering based on AnMsrA) is implicated in the catalysis of sulfoxide reduction, and its side chain has been identified as a critical catalyst, but not in substrate binding. In agreement with their finding, our data revealed that Glu99 is of central importance for the activity of AnMsrA. Similarly, Antoine et al. (2006) showed that substitution of Glu99 by Asp strongly increased the Km value of *N. meningitidis* MsrA from 0,6 mM to 161 mM. The mutation Glu99Gln decreased AnMsrA activity toward Val-, Phe- and the right-sided Asp-containing peptides 2-3-fold.

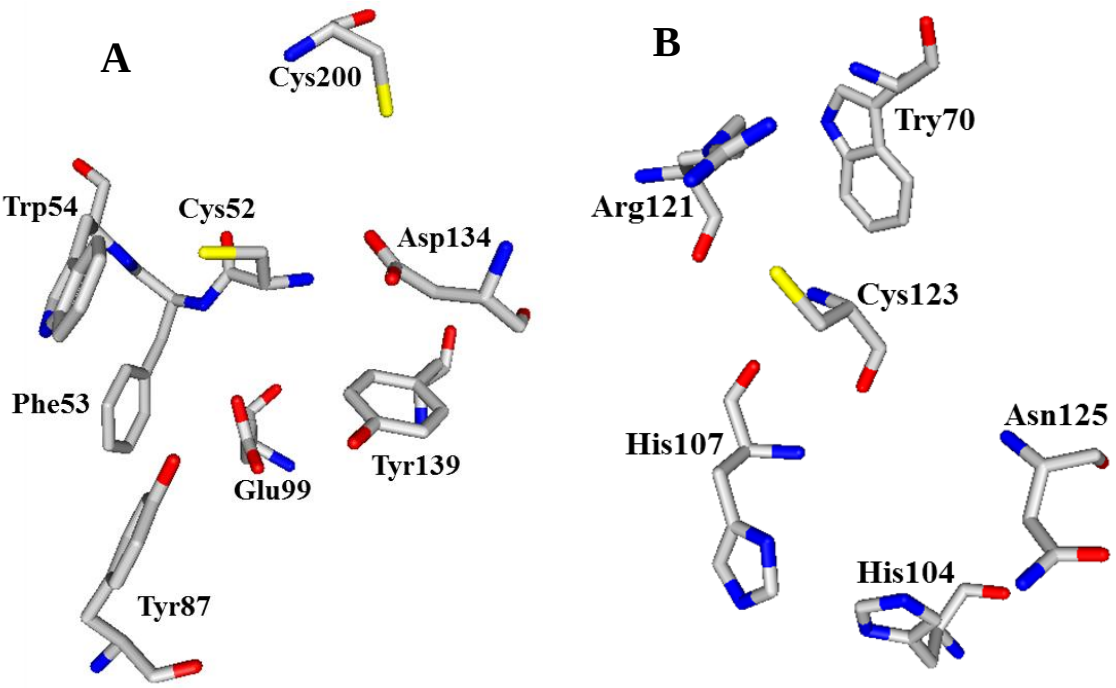

Fig. 38. Active-site model of *A. nidulans* MsrA and MsrB. (A) Stick representation of AnMsrA residues Cys52, Phe53, Trp54, Tyr87, Glu99, Tyr139, Asp134, and Cys200. **(B)** Stick representation of AnMsrB active site residues Cys123, Arg121, His104, His107, Try70 and asn125. Atom color as follows: carbon atoms in gray; nitrogen in blue; oxygen in red and sulfur in yellow. This figure was created using the program PyMOL.

However, decreasing the activity 11-16 fold toward Asp- and the left-sided Asp-containing peptides, respectively, suggesting that Glu99 has a role not only in the catalysis of MetO reduction but also in substrate binding and discrimination. The activity of Asp134Asn decreased a 15-fold toward Asp-containing peptide. Gand and others (2007) showed that while the side chains of Asp134 (AnMsrA) and Tyr139 participated in the substrate binding, they did not discriminate in terms of binding between a MetO either engaged in amide bonds or with amino and carboxyl groups free. In contrary to Gand et al. (2007), our data strongly suggest that Asp134 also has a role in the substrate binding and discrimination.

The data also showed that, along with Glu99Arg, substitution of Asp134 for Arg also resulted in complete loss of AnMsrA activity, suggesting that AnMsrA reducing Met-S-O through an acidic-dependent mechanism. Double substitution of acidic residues (Glu99 and Asp134) for uncharged hydrophilic residues (Gln and Asn) respectively, also caused complete loss of AnMsrA activity. Furthermore, exchange of Glu99 and Asp134 also caused complete loss of activity, indicating that they not only participate in the substrate binding and discrimination but also they might be involved in the structure stability of AnMsrA. More interestingly, substitution of Asp134 by Glu unexpectedly increased AnMsrA activity toward the Asp- and

Glu-containing peptides by 2- and 2.5-fold, respectively, whereas the activity was similar to the wild-type enzyme toward the rest of peptides. The results suggest that the presence of two Glu residues on positions 99 and 134 clearly enhanced the catalysis of Met-S-O by AnMsrA and disrupt the discriminating point at the active site. At the same time, these data pinpoint Asp134 to be the residue that might be responsible for protein-substrate selectivity for AnMsrA.

4.7 Role of Arg121 in the active site of AnMsrB

In comparison with MsrA, literature information about the active site of MsrB and the residues that are involved in the catalysis of Met-R-O is less. Previous studies showed that MsrA and MsrB have similar catalytic mechanisms with asymmetry of the active site, although the residues involved in the catalysis are different (Lowther et al., 2002; Antoine et al., 2006; Neiers et al., 2007). In contrast to MsrA, our data showed that AnMsrB activity is less sensitive to an acidic context. As mentioned above, the crystal structure comparison revealed a mirror-like relationship between MsrA and MsrB active sites (Fig. 38), except that MsrA has acidic residues (Glu99 and Asp134 AnMsrA) whereas MsrB has basic residues (Arg121, His104 and His107 AnMsrB).

In order to investigate the role of the Arg residue in the AnMsrB active-site, we mutated this residue to Asn or Asp. The substitution of Arg121 for Asn or Asp showed similar effects on AnMsrB activity except toward DmD peptide; Arg121Asn exhibited 10-fold decrease while Arg121Asp exhibited 20-fold decrease in AnMsrB activity. It showed similar activity of wild-type AnMsrB toward Met-R-O in the basic or uncharged residues containing peptides; similarly substitution of Arg for Leu had no effects on the activity of MsrB from *N. meningitidis* (Neiers et al., 2007). However, the activity decreased variably toward the acidic-containing peptides especially the Asp-containing peptide. Arg116 (equivalent to Arg121 in AnMsrB) showed no role in the activation of the catalytic Cys (Cys123 AnMsrB) for *N. meningitidis* MsrB and mutated to Leu showed no effects on the enzyme activity (Neiers et al., 2007). In contrast, our results showed that substituting Arg121 for Asp or Asn drastically decreased the AnMsrB activity toward the peptides with acidic residues especially substitution for Asp. These data suggest that Arg121 is directly or indirectly involved in the catalysis of Met-R-O and Arg121 is the discriminating residue for protein substrate selectivity of AnMsrB.

5 Outlook

The results presented in this thesis established *A. nidulans* as a new model organism for studies on Msr enzymes and their physiological relevance. Future work may focus on the intracellular localization of all these enzymes and their specific roles in oxidative protection. This can be considered as a foundation for investigating all three classes of Msr enzymes in multicellular organism. The genus *Aspergillus* includes human and plant pathogens as well as beneficial species used to produce foodstuffs and industrial enzymes. For example, *A. fumigatus* is a deadly pathogen of immunocompromised patients; *A. flavus* is an agriculturally important toxin producer; *A. niger* and *A. oryzae* are used in industrial processes.

This thesis provided first evidence for the variable relevance of individual Msr enzymes depending on the type of oxidative stress. The analysis of this connection between stress different stress factors and the set of repair enzymes will be interesting in the future. Also more studies would be required to fully understand the mechanism underlying the difference in peptide selectivity of MsrA and MsrB enzymes.

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7 Appendix

Abbreviation

wt	Wild type
mt	Mutant
Msr	Methionine sulfoxide reductase
SEM	Standard Error of the Mean
ROS	Reactive Oxygen Species
DTT	Dithiothreitol
CaM	Calmodulin
A.	*Aspergillus*
AMM	*Aspergillus* minimal media
Amp	ampicillin
β-GAL	β-galactosidase
bp	base pair
°C	degree Celsius
cDNA	DNA complementary to mRNA
Δ	deletion
Da	dalton
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
U	unit of enzyme activity
v/v	volume per volume
E.	*Escherichia*
e.g.	for example
et al.	and others
g	gram
h	hour
HPLC	high performance liquid chromatography
RP-HPLC	Reverse phase HPLC
i.e.	that is
kbp	kilobase pairs
kDa	kilodalton
l	litre
M	molar
min	minute
mRNA	messenger RNA
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
SD	standard deviation

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2. Moghaieb REA, MA Elawady, **Rabab G. El-Mergawy**, SS Youssef and AM El-Sharkawy (2006). A reproducible protocol for regeneration and transformation in canola (*Brassica napus* L.). African Journal of Biotechnology Vol. 5 (2), 143-148.

3. Youssef SS, REA Moghaieb, **Rabab G., El-Mergawy** and AM El-Sharkawy (2007). Genetic markers associated with salt tolerance in canola (*Brassica napus* L.). Arab Journal of Biotechnology, 10(1):143-154.

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2. Zhu Q, **Rabab G. El-Mergawy**, SH Heinemann, R Schönherr, P Jáč and GK Scriba (2014). Stereospecific electrophoretically mediated microanalysis assay for methionine sulfoxide reductase enzymes. Anal Bioanal Chem. 406(6): 1723-1229.

3. **Rabab G. El-Mergawy**, SH Heinemann, and R Schönherr (2014). Peptide specificity of MsrA and MsrB from human and *A. nidulans*.

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Ehrenwörtliche Erklärung

Ich erkläre, dass

- mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist.

- ich die Dissertation selbst angefertigt habe. Alle benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen sind in der Arbeit an entsprechender Stelle angegeben.

- ich nicht die Hilfe eines Promotionsberaters in Anspruch genommen habe und dass im Zusammenhang mit dieser Arbeit keine Zahlungen oder geldwerten Leistungen an Dritte gegangen sind.

- ich diese Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe.

- ich bei keiner anderen Hochschule eine Dissertation eingereicht habe.

Jena, März 2014

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